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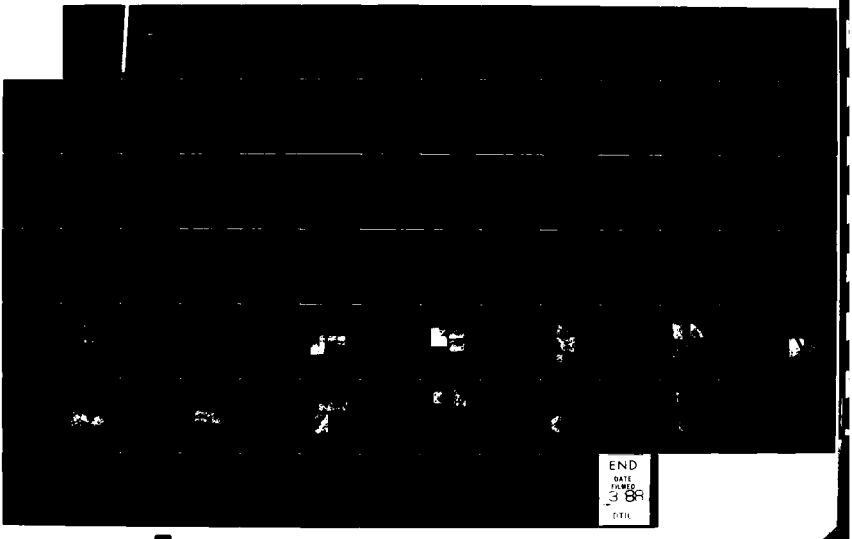

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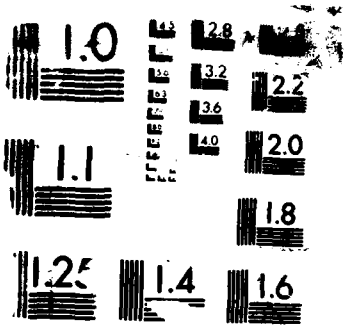

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EFFECTS OF CHRONIC PYRIDOSTIGMINE ADMINISTRATION ON MUSCLE FATIGUE AND MORPHOLOGY

Final Report

Rachelle N.S. Fishman, Ph.D.
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Pyridostigmine, a reversible anticholinesterase is used prophylactically against subsequent exposure to organophosphates. Yet, there is evidence from animal studies that reversible anticholinesterase agents can induce structural and functional abnormalities in the neuromuscular system. The damage appears to require a threshold exposure in time and dose, and can be prevented by prior denervation of the muscle, implying that the origin of the damage stems from the extended presence of acetylcholine (ACh) released into the neuromuscular junction from the presynaptic nerve, resulting in excessive activation of proteases by the influx of calcium at the motor endplate.

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Since activity of the muscle could change synthesis and turnover of ACh, it could also modulate damage and repair in the nerve-muscle system related to cholinesterase inhibition.

This study provides evidence of interaction between effects of treatment with pyridostigmine and strenuous exercise.

Pyridostygmine was given per os to rats from 3 to 28 days. Behavioral performance on a high intensity exhaustive exercise program (running on a high speed treadmill for 1 hour per day) was assessed. Electromyographic, myoneural response to high frequency electrophysiological stimulation was measured; and ultrastructural aspects of muscle and neuromuscular junction were evaluated.

The effect of pyridostigmine (that produced an average of 40% inhibition in whole blood AChE activity) was dependent on the state of pretraining of the rat. When rats were simultaneously trained and treated from 2 to 4 weeks, no major behavioral deficits were found in treated rats compared to running controls (Paradigm I). When rats were pretrained to run and then treated, the variance of their individual running performances increased markedly; that is, they performed less consistently (Paradigm IIa). When the drug was stopped but running continued, performance generally improved with no lingering drug effect (Paradigm IIb, c). Short periods of treatment appeared to improve performance. Treating during the first three days of training increased consistency of running and endurance, while longer treatments tended to depress performance.

These behavioral findings were consistent with acute in situ electrophysiological measurements. The treated animals showed less fatigue in electromyographic response in comparison to nontreated control rats, and there was no evidence of conduction block.

Fine structural examination of the gastrocnemius muscle revealed focal damage at random neuromuscular junctions and in subjunctional muscle fibers. Few muscle fibers were affected relative to the total number of fibers examined. Increasing the treatment length appeared to have a decreasing effect on overall damage. Exhaustive running was itself damaging but morphology of treated-running rats seemed less affected.

Thus, the neuromuscular system appears to show an ability to adapt to experimental drug and exercise conditions in a dynamic manner.

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EFFECTS OF CHRONIC PYRIDOSTIGMINE ADMINISTRATION ON MUSCLE FATIGUE AND MORPHOLOGY

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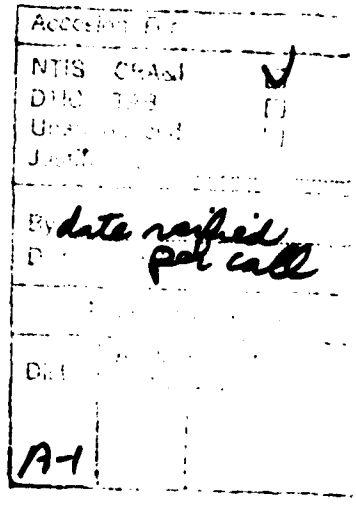

SUMMARY

Pyridostigmine, a reversible anticholinesterase is used prophylactically against subsequent exposure to organophosphates. Yet, there is evidence from animal studies that reversible anticholinesterase agents can induce structural and functional abnormalities in the neuromuscular system. The damage appears to require a threshold exposure in time and dose, and can be prevented by prior denervation of the muscle, implying that the origin of the damage stems from the extended presence of acetylcholine (ACh) released into the neuromuscular junction from the presynaptic nerve, resulting in excessive activation of proteases by the influx of calcium at the motor endplate.

Since activity of the muscle could change synthesis and turnover of ACh, it could also modulate damage and repair in the nerve-muscle system related to cholinesterase inhibition.

This study provides evidence of interaction between effects of treatment with pyridostigmine and strenuous exercise.

Pyridostygmine was given per os to rats from 3 to 28 days. Behavioral performance on a high intensity exhaustive exercise program (running on a high speed treadmill for 1 hour per day) was assessed. Electromyographic, myoneural response to high frequency electrophysiological stimulation was measured; and ultrastructural aspects of muscle and neuromuscular junction were evaluated.

The effect of pyridostigmine (that produced an average of 40% inhibition in whole blood AChE activity) was dependent on the state of pretraining of the rat. When rats were simultaneously trained and treated from 2 to 4 weeks, no major behavioral deficits were found in treated rats compared to running controls (Paradigm I). When rats were pretrained to run and then treated, the variance of their individual running performances increased markedly; that is, they performed less consistently (Paradigm IIa). When the drug was stopped but running continued, performance generally improved with no lingering drug effect (Paradigm IIb, c). Short periods of treatment appeared to improve performance. Treating during the first three days of training increased consistency of running and endurance, while longer treatments tended to depress performance.

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decreasing effect on overall damage. Exhaustive running was itself damaging but morphology of treated-running rats seemed less affected.

Thus, the neuromuscular system appears to show an ability to adapt to experimental drug and exercise conditions in a dynamic manner.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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Figure 2 A. AChE activity measured in mmoles ACh hydrolyzed per ml whole blood per minute. Baseline response, prior to separation of rats into treatment groups. Overall: 750 ± 111 (N = 150).

B. Following pyridostigmine (Pyrido.) treatment and treatment with running (Pyr.Run), there was a significant inhibition in AChE activity ($p < 0.01$). Measurements were pooled for each rat relative to its own AChE activity prior to treatment. The control and untreated running groups (Con.Run) continued to show wide variance without significant inhibition.

Figure 3. Running performance of pyridostigmine-treated and non-treated rats in paradigm I. To show differences within the hour of running, shocks per minute were meaned separately over 15 minute intervals.
* = $p < 0.05$; ** = $p < 0.01$; *** $p < 0.001$, for all figures and tables (Kruskall-Wallis ANOVA).

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Figure 19. Electron micrograph, X-S, of gastrocnemius muscle of a 28-day pyridostigmine-treated running rat. Note the generally intact muscle. Dense staining vesicles and inclusions are scattered throughout the post-junctional folds (arrows). The sarcoplasmic reticulum (star) is distended in four places.

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INTRODUCTION

Statement of the Problem

Pyridostigmine, a quarternary carbamate, is an anticholinesterase (antiChE) agent which is effective in protecting experimental animals against lethal doses of rapidly aging, irreversible, organophosphate (OP) antiChE agents (1, 2). Pyridostigmine is routinely used to treat myasthenia gravis in human beings, and pyridostigmine has been considered prophylactic against subsequent exposure to OPs (3). The intended routine use of pyridostigmine prophylaxis in human beings must be balanced against evidence from animal studies that reversible antiChE agents can themselves induce structural and functional abnormalities in muscle and neuromuscular apparatus (4-13).

This study was designed to provide correlative evidence from behavior (running), electrophysiology (acute stimulation), and morphology (neuromuscule ultrastructure) following chronic exposure to pyridostigmine in order to delineate areas needing further investigation.

Pyridostigmine was given to rats from 3 to 28 days to evaluate different schedules of high intensity exhaustive exercise performance (running on a treadmill for 1 hour per day), acute myoneural response to high frequency electrophysiological stimulation, and ultrastructural morphology of muscle and neuromuscular junction.

Background

Many studies have indicated that structural abnormalities in muscle and/or neuromuscular apparatus develop after exposure to antiChE agents (5, 7, 9, 11-16). The damage appears to require a threshold exposure in time and dose (8) and can be prevented by prior denervation (9), implying that the origin of the damage stems from the extended presence of acetylcholine (ACh) released into the neuromuscular junction from the presynaptic nerve. In consonance with this, degenerative changes in muscle fibers were focused at subjunctional areas. The myopathy itself is thought to result from excessive activation of proteases by the influx of calcium at the motor endplate because of extended activity of ACh in the neuromuscular cleft (12, 17).

Muscle activity, which would change synthesis and turnover of ACh, might modulate the degrees of damage and repair in the nerve-muscle system after antiChE treatment. The actions of antiChE agents are not restricted to causing accumulation of ACh in the synaptic cleft (18-20). Long-term treatment with antiChEs (or other agents that prolong the lifetime of the ACh molecule) have been found to result in tolerance, decreased sensitivity of postsynaptic ACh receptor-ion channel complexes (21-23), and change of presynaptic conduction. Long-term treatment led to loss of some force production in muscles when stimulated at high frequencies (24, 25).

The research interest in changes in the neuromuscular apparatus following antiChE treatment has not been matched by a similar effort to examine motor activity, although behavioral studies (changes in motor performance during and following chronic exposure to antiChE drugs) are essential to evaluate the relevanece of neuromuscular deficits per se. One recent study (26), using soman, physostigmine and pyridostigmine at doses that did not produce overt symptoms of cholinergic crisis, did show that motor behavior requiring more coordination (e.g., hurdle-stepping) was impaired, while spontaneous activity was unaffected.

Rationale

This study was intended to examine the interaction of pyridostigmine and strenuous exercise.

The rat was chosen for this study because at reasonable investment, relatively large numbers of animals can be trained to run on a treadmill, and the few existing studies of antiChE agents on motor behavior utilized rats (26-30). We tested the effect of pyridostigmine on rats subjected to intensive exhaustive exercise. Using treadmill locomotion, we quantified changes in running performance (see Experimental Methods, below). Other studies (31) support the use of our treadmill speeds (up to 31.3 m/min) which constitutes a full run for the rat and a test of maximal effort.

Rats were dosed per os ad libitum with pyridostigmine in water after we had established in pilot studies the longevity of the molecule in water, and that the rat's water consumption per day per gram body weight was consistent. In addition, human patients typically receive the drug per os. The pyridostigmine dose was chosen after pilot studies had shown that 12.5 mg/kg/day in drinking water produced about 40% inhibition of acetylcholinesterase (AChE) (the degree of inhibition necessary for protection against OPs; personal observatirons, M.Chipman).

The electrophysiological program of stimulation was chosen to test the maximal output of the running muscles (triceps surae), which includes the gastrocnemius, plantaris and soleus muscles (32).

Morphological examination concentrated on ultrastructural analysis to localize the specific sites of drug-induced changes and to estimate the extent of the changes produced. The gastrocnemius muscle was examined because it is the primary leg flexor used in running.

EXPERIMENTAL METHODS

Experimental Animals

Female Fischer rats were studied. All rats within the experimental protocol were weighed every 3 to 10 days, depending on their treatment designations, and again on the day of sacrifice.

Rats were divided into treatment groups: control rats, given no pyridostigmine (49); pyridostigmine-dosed (56); pyridostigmine-dosed and run (24), paradigm I, Figure 1, run without pyridostigmine (18), paradigm I, Fig. 1. In addition, 20 rats were tested on a multiple schedule of running with and without having been dosed, paradigms II and III, Figure 1.

Behavioral Methods: Training and Analysis

Rats were run for one hour per day on a treadmill (Figure 1). The treadmill is approximately 1.5 rat lengths or about 30 cm long. When the rat tired and could not keep up the running pace with the moving treadmill, it fell back against the rear wall, and closed a shoking electric grid circuit which delivered ca. 0.5-5 ma shock.

Rats were trained and run in 3 different paradigms to compare overlapping dosing and training effects. In paradigm I rats were dosed with pyridostigmine simultaneously with their training. Rats were run for the first 2 days at a treadmill speed of 15 m/min. Speed was increased to 20.4 and to 28 m/min until a maximum speed of 31.3 m/min was attained by the 5th day of training. On all days, run time was a full 60 minutes. Rats were run for a total of 3, or 7, or 14, or 21, or 28 days. During each 60 minute run period, the treadmill was stopped for less than 1 minute after every 15 minutes to permit re-wetting of the animal for shock conduction; running was then continued at the maximum speed for that hour.

Paradigm II was identical to the foregoing (paradigm I), but the maximum treadmill speed attained, on the 5th day of training, was 28.6 m/min.

In paradigm III, the training period was more gradual and was extended to 14 days before attaining the maximum treadmill speed. Initial treadmill speed was 15 m/min and the total initial run time was restricted to 15 minutes. Track speed and run time were increased daily to a maximum treadmill speed of 28.6 m/min and a total run time of 60 minutes by day 14.

Rats were run 6 of 7 days per week. In certain animals a record of body temperature was taken before the running session and after the 60 minute run. Similarly, weight change differences between exercising and sedentary rats were monitored. The analysis of this data was considered beyond the scope of the current report (M.C.) so it is not presented here.

It could be incorporated into a more extensive study of exercise induced thermogenesis.

The record of shocks is a measure of the rats' ability to maintain pace over time, or conversely, of behavioral endurance. The shocker was attached to an electromechanical counter and the number of shocks was accumulated per 15 minute period for each daily hour of running. In paradigm III (Figure 1), the training period was extended to over 14 days with a gradual increase in length of time and speed of running.

Behavioral performance was estimated for each rat by:
(1) Shocks per minute for 15, 30, 45 and 60 minute segments
(2) Shocks per minute ratio 60:30
(3) Shocks per minute ratio 60:15
(4) Total shocks/total run time
(5) Total shocks/30 to end
(6) Total shocks/45 to end.

Computed variables 4-6 included days of rats which collapsed during their training/running, that is, ran less than 60 minutes (also see Table 2). Collapse periods were treated as missing data.

The data were analyzed for each rat for each day by multivariate analysis of variance (ANOVA), then data regrouped for ANOVA over weeks, also deriving a statistic, the runweek, counting days after the rest day, over treatments and weeks.

For each running rat, the whole blood AChE activity and its inhibition were measured for ANOVA and Pearson's correlation coefficient (r).

The rats were compared within and between schedules. In paradigm I (see Figure 1), the training period overlapped with the drug dosing period and consisted of 2 days at a slow pace (15 m/min), then 20.4, then 28 to 31.3 m/min by the 5th day. In paradigm III, the rats were pretrained to run 60 minutes at maximal speed over 2 weeks; dosing began after 28 days of running. Each rat's performance was normalized according to the formula:

$$Y(i)_{i=1 \text{ to } n} = S(i)_{i=1 \text{ to } n} \Big/ \text{SIGMA } S(i)_{i=14}$$

where Y(i) = normalized shock index for day; s = number of shocks per minute in the period of interest (viz. list of variables 1 through 6, above). In addition to normalization by the mean of days 14-28, the median was determined and then scored by rank difference.

In paradigms I and II, the rats were stopped every 15-30 minutes for less than 1 minute and were re-wet on their back, rump and tail (for conduction of shocks). In paradigm III, Grass conductive paste was applied

to the same parts of the rat's body, above, and neither re-wetting nor stopping was necessary for the entire 60 minutes.

Data were analyzed in a line-by-variable matrix on a VAX 11/780 computer, using the Statistical Program for the Social Sciences (SPSS) routines for parametric and non-parametric statistics, and programs written in FORTRAN by E.Fagin. A statistic was derived (E.Fagin) to estimate regression slope of performance along time axis (by calculating median slope for each animal for each variable), based upon the algorithms of Thiele and Hollander (33).

Drug Delivery and Schedule of AChE Determinations

In pilot studies, we compared drug delivery of identical doses of pyridostigmine (12.5 mg/kg/day) by (1) Alzet pump, (2) subcutaneous injection, (3) intubation by gastric cannula, (4) water deprivation and feeding, and (5) continuous availability ad libitum in drinking water, and (6) undosed but equivalently treated controls, respectively. The results of the pilot study showed an average inhibition (N=4 each treated and controls) of 10 ± 1.5% for Alzet pump, 24 ± 1.6% for subcutaneous injection, 24 ± 3.1% for drug in water given every 6 hours, and 47 ± 3.1% for water administered ad libitum. The gastric cannula method was eliminated during the pilot study because in our hands it required light ether anesthesia four times per day, and it is known that exposure to anesthetics changes the character of neuromuscular responses (34).

Since pyridostigmine is generally delivered per os in humans, and it has a short half-life (viz. 35) an oral route was preferred.

The ad libitum drinking method was adopted. Water intake of rats was monitored prior to dosing (N=26). These included male, female, caged, exercised, juvenile and adult rats ranging in weight from about 125-300 g. Rats drank at a regular rate (15-25 ml/day), which varied within this range depending upon activity, age, and weight. Thus, these variables were normalized. Knowing the rats' body weight and water consumption, the desired dose in mg/kg of drinking water could be obtained. Dosing did not change the drinking rate which was measured before and during dosing.

A small dose experiment using 1.25, 2.5, 5.0, 10.0 and 12.5 mg/kg (N=4 each) of pyridostigmine for 3 and 7 days resulted in a variable inhibition relative to baseline blood AChE activities. 12.5 mg/kg was most consistent in producing 40% inhibition and this dose was consistent with that recommended by M. Chipman (personal communication).

Rats were assayed for AChE levels on day 0 (prétreatment) and on the day of sacrifice by bleeding from the tail.

Acetylcholinesterase Assays

AChE assays were performed by the radiometric method of Johnson and Russel (36), using (^{3}H-acetyl)-choline as the substrate.

Blood (150 µl) was drawn from the tail into 1.5 ml tubes containing 20 µl cold heparin sulfate. Cold 1% Triton-X-phosphate buffer, pH 7.3 (130 µl), was added immediately; samples were gently mixed, then frozen at -80°C until transported (in liquid N^2) to the laboratory of Dr. Israel Siliman of the Weitzmann Institute, Rehovot, Israel. Each sample was divided for replicates and assayed, including simultaneous assay with BW 284-C51 dibromide to inhibit pseudocholinesterase.

Multiple assays of blood samples (15-20) taken from the same rat (N=4) by heart puncture (after pentobarbital, 50 mg/kg anesthesia) varied by 9-15%.

Morphological Methods

Rats were weighed and anesthetized intraperitoneally (ip) with sodium pentobarbital (50 mg/kg). For light and electron microscopy, rats were perfused with Karnovsky's fixative in 0.1 M phosphate-buffered saline (PBS), 4% sucrose, pH 7.4. Gastrocnemius and soleus muscles were dissected free. The soleus muscles were weighed. Muscles were pinned at rest length in cold fixative overnight, then rinsed thoroughly with PBS, dissected into bundles, and stained to enable visualization of neuromuscular junctions, with bromoindoxylacetate stain for AChE (modified from Gomori, Ref. 37). Treated and control muscles were routinely stained in the same dish for comparison. Tissue containing the motor endplates was dissected for embedding for thin sectioning; tissues were post-fixed in 2% OsO_4 in PBS for 1 hour, 4°C, in the dark, stained en bloc with 0.5% uranyl acetate, dehydrated in a graded series of ethanols, and embedded in Spurr. Semithin sections (1 µm) were cut and stained with toluidine blue borax to locate the endplates.

Ultrathin sections (60-100 nm) on copper grids were stained with saturated uranyl acetate and lead citrate. Sections were cut at varying depths through the motor endplate and surrounding sarcoplasm.

For histochemical fiber typing, the method of Padykula and Herman (38) for myofibrillar ATPase, at pH 9.4 with preincubation at pH 4.3 (39) was used. For classical analysis of the drug effect on muscle fiber type ratios, at least 300 muscle fibers were counted in each muscle of each control and treated rat (N=4). To improve the analysis (40), fiber types were counted within separate muscle fascicles and a ratio of the averages of these counts was calculated.

Electrophysiological Tests

Rats were anesthetized with pentobarbital sodium (30-50 mg/kg, ip). The sciatic nerve was exposed near its emergence from the sciatic notch. Stimulating electrodes were placed under the nerve and covered with saline-soaked cotton.

The achilles tendon was cut (with tendons of triceps surae, gastrocnemius, plantaris and soleus muscles) and attached to a Grass FT03 force displacement transducer. The knees and ankles were anchored in place. Since the muscle was not cut, its blood supply remained intact. Concentric electromyographic (EMG) recording needles were inserted into the gastrocnemius muscle.

A 4-channel TECA MSG equipped with an ADG strain gauge amplifier converted the EMG and tension signals and recorded them simultaneously on photographic recording paper. Stretch and tension were calibrated into mg per ml; measurements of signal amplitudes and duration were taken from the photographic recordings during the first and last stimulation sessions as an internal comparison of fatigue with electrical stimulation.

The schedule of stimulation is shown in Figure 9 for the following sciatic nerve stimulations:

(1) A single reference shock

(2) Continuous train of 50 Hz shocks for 10 seconds (preliminary studies indicated that with this stimulation, reproducible decremented signals indicative of muscle fatigue occurred in normal rats)

(3) Twenty minutes after administration of the stimuli described in (2), 100 Hz 0.1 second duration tetanic shocks (N=10-15) every 20 seconds. (This procedure imitates the bursting activity of motor neurons; signal decrements are indicative of neuromuscular junction blockade.) The nerve was stimulated with 0.1 msec duration supramaximal pulses. Short trains of either 8, 16 or 32 pulses at 100 Hz were given every 10 seconds for 10 minute sessions. Between 10 minute sessions, the preparation was allowed to rest for 10 minutes.

The power output of the muscles was recorded at the beginning and the end of each session (each time average of 3 trains was calculated). A fatigue ratio (power output at the end of session divided by that of the beginning) was then calculated for each of 8, 16, or 32 stimuli per session in each animal.

RESULTS

Acetylcholinesterase Activity in Serum. AChE activity was measured in whole blood prior to treatment (Table 1, Figure 2A) and after treatment (Table 1; Figure 2B). At all times, there were large interindividual variations in AChE activity in whole blood, despite which pyridostigmine treatment resulted in significant ($p < 0.01$) or 40% or greater depression relative to respective activity before treatment.

AChE inhibition was negatively correlated with the length in days of pyridostigmine treatment ($r^2 = -0.76$; $p < 0.001$) and of pyridostigmine treatment-with-daily-exhaustive running ($r^2 = -0.65$; $p < 0.01$). Neither the Pearson's correlation for cage-held nontreated rats ($r^2 = 0.001$) nor that for running untreated rats ($r^2 = 0.020$) was significant.

Survey of AChE Staining of Motor Endplates of Muscle Fibers. Gastrocnemius muscles were stained for localization of motor endplates (neuromuscular junctions) using bromoindoxylacetate. When pyridostigmine-treated and untreated muscles were stained simultaneously in the same dish with the bromoindoxylacetate stain, the time to stain was 25% to 75% longer in treated muscles than in controls, while maximum stain intensity was not greater than that of the controls. More notable was the striking difference between soleus and gastrocnemius muscles: the soleus endplates stained two to three times more slowly than those of the gastrocnemius, and the maximum stain intensity was always pale, faint, delicate, and opalescent in contrast to the dense, dark, prominent staining of the gastrocnemius endplates.

Morphological Results

Histochemical Findings. Following staining with alkaline ATPase preincubated at pH 4.3 of frozen muscle sections from treated and control rats, the overall number of heavily, weakly and intermediate stained muscle fibers was counted. To improve the analysis, the fiber types were counted within separate muscle fascicles and means and ratios were calculated.

In soleus muscle from untreated rats, the weakly staining fibers were predominant, 76% of total or about 5 times that of the heavily staining fibers (Table 5). In gastrocnemius muscle, fibers types were more evenly distributed, and weakly staining fibers composed about 35% of total fiber population. After treatment with pyridostigmine, light-staining fibers disappeared as a class in both soleus and gastrocnemius muscles. In soleus, the intermediate muscle fiber class increased by an order of magnitude, while in gastrocnemius the loss of light-staining fibers was not accompanied by a change in intermediate staining fibers and about 20% increase in dark staining fibers relative to pre-treatment values for each class (Table 5).

Synapse Quantification. Preliminary counting of the number of axon terminals per synapse showed no notable differences among treatment groups relative to the controls (total number of synapses = 113; Table 6). There

was some indication of decreased number of axon terminals per synapse with early pyridostigmine treatment, but in too few specimens to conclude that this was a valid result. Moreover, the variance was quite high within and among individual synapses and muscles.

Muscle and Neuromuscular Damage. Exercise and pyridostigmine treatment affected muscle mass per se. The ratio of soleus weight:body weight was significantly greater in the pyridostigmine-treated group than in the control group at 14 days and significantly less in the treated group than in the control group at 21 days (Table 7).

The most obvious change in muscle morphology was the appearance of damaged (cystic, broken) mitochondria. However, not all myofibers within the same muscle were equally affected, nor all individuals within a treatment group. The wide range of damage and change from the control muscle is shown in Figures 11 through 21.

In the electron microscope, the postjunctional folds fill with electron-dense AChE stain (Figure 11). Many, but not all junctions from control rats were so stained. In contrast, less than 0.05% of junctions from pyridostigmine-treated rats were stained.

The muscles of control, non-running non-treated rats were indistinguished. Mitochondria (m) were normal and compact; sarcoplasmic reticulum (s) and subjunctional areas were filled with glycogen (g); postjunctional folds varied in density and regularity (Figure 12).

Treating with pyridostigmine produced a wide variety of changes in nerve, muscle, and neuromuscular synapse (Figure 13). These included disrupted and broken mitochondria, distended sarcoplasmic reticulum; a variety of inclusions and vacuoles in the axon terminal; some sparse synaptic vesicles; and both hypo- and hyperplasia of postjunctional folds. The extent of variation was great and that between animals was equaled by that between synapses and muscle fibers within the same animal.

Both treating with pyridostigmine-and-exhaustive-running resulted in even greater disruption especially in the subjunctional muscle fiber which was distended and filled with disrupted mitochondria. In many junctions, axon terminals were missing, leaving empty synaptic gutters. The extent of variations between fibers was notable (viz Figure 14). Even without pyridostigmine, running exhaustively produced widespread mitochondrial damage (Figure 15).

Treating for longer periods of time with pyridostigmine produced even greater variation in the morphology of the neuromuscular junction, axon terminals, and muscle itself (Figures 16, 17, 18). The damage to the sarcoplasmic reticulum and to the muscle mitochondria, which are sometimes dense in the subjunctional space, was subtle.

Continued treatment with pyridostigmine combined with exhaustive exercising appeared to result in less muscle damage (Figure 19) than longterm exhaustive exercising alone (Figure 20).

Evidence of damage was also found outside the nerve-muscle synapse in treated, running rats (Figure 21).

Running Performance and Pyridostigmine Treatment

Measurements of performance during high speed interrupted treadmill running revealed that the tests were exhaustive (paradigm I), as indicated by the frequent occurrence of collapsing and the inability to complete the 60 minute daily running session (Table 2). An average of 52% of the rats collapsed at least once during running (Table 2). There were only minimal indications that treatment with pyridostigmine changes maximum performance output (Table 2). Rats that eventually collapsed before the 60 minutes running sesson was over received significantly more ($p < 0.005$) shocks per minute before collapsing than those rats which ran the full 60 minutes (Table 3). Only within the third 15-minute period of the running hour was there an indication that pyridostigmine affected endurance. Only during this period, was a significant difference ($p < 0.05$) in distribution of shocks due to treatment found. Treated rats received fewer shocks in both collapse and non-collapse categories.

Rats were run and treated simultaneously with no pretraining period. The analysis showed that treatment generally decreased shocks per minute within the first 3 days of treatment and running only (Figure 3); this conclusion was reinforced by a ranked comparison of performance during each 15 minute interval, meaned across weeks of the experiment (Table 4, Mann-Whitney, $p < 0.05$, analysis of slopes). Untreated rats performed more consistently during the entire hour of running than treated rats (Table 4). During the first 2 weeks of running, there was a significant increase in numbers of shocks received by treated rats at the end of the 60 minutes of running, compared to the beginning ($p < 0.04$). The treated rats performed worst during the 0-15 minute (warm-up) period, improved and then tired towards the end of the hour. After 2-3 weeks of running, the treated rats progressively improved during the 60 minute running session. The need for a warm-up was still present within the treated group, but then they progressively improved towards the end of the hour ($p < 0.04$, Friedman 2-way ANOVA) (Table 4; Figure 4). Linear regression analysis indicates that for the 30 and 45 minute periods, both treatment and running experience tended to decrease the number of shocks per minute ($p < 0.001$).

Rats did not run every day but had 1 rest day in 7 days of running. The performance of the treated rats was generally improved after the rest day (Figure 5). The difference between treated and untreated runners was apparent over all 15 minute periods ($p < 0.001$, 2-way ANOVA), although the effect of rest on improved performance within the hour of running was only marginally significant, and became so only after 4 weeks of running ($p < 0.05$, ANOVA; Figure 6).

The change in shocks received during running showed a significantly improved performance during days 1 to 28 regardless of treatment or mode of running ($r^2 = 0.31$; $p < 0.04$; see also Figures 3 and 4).

Regardless of drug treatment or of schedule of training (10 to 60, Figure 1), rats attained a post-training plateau of performance between days 15 and 28 (no effect of day on any 15 minute interval of running; ANOVA, $p > 0.05$). Comparing this plateau period across all treatments revealed that both the untreated and treated runners which had been trained gradually (groups 60, 61) received significantly fewer shocks per minute ($p < 0.01$, ANOVA) than the other comparable groups. This improved performance was emphasized during the endurance phase of testing, after 30 minutes of running. In contrast, the untreated intermittent runners (group 10) had the worst performance-shock index of all other groups (group 20) ($p < 0.05$, ANOVA; Figure 7); treating these runners with pyridostigmine improved performance ($p < 0.05$, ANOVA; Figure 7). After the 15-28 day plateau in performance of the intermittently trained runners, when treatment of a running group was changed from untreated to pyridostigmine-treated (see Figure 1, groups 30-31 and 50-51), the performance-shock index worsened or remained unchanged (Figure 8). When the treatment was stopped and running continued (groups 40-41;51-52) the performance-shock indexes worsened (Figure 8). In contrast, gradually trained runners treated with pyridostigmine (61) during the post-plateau test period (days 29-42) continued to consistently obtain significantly fewer shocks than other equivalent groups (especially 51, 31) and particularly during endurance phase of the running period after 30 minutes of running (Figure 7).

Physiological Results

The results of a typical experiment on rats treated with pyridostigmine for 4 weeks are shown in Figures 9 and 10. The EMG recordings do not show evidence of a conduction block in the nerve, although some minimal electrical decrement can be seen (Figure 9). There is some slight reduction in the amplitude of the action potential taken from the last train as compared to the first. Simultaneous isometric tension recordings show that the system maintained tetany during each train with no indication of becoming unfused (Figure 9). Fully fused tetany appeared only at stimulation rates higher than 60 Hz, at which frequency maximal tetanic tension was also produced. The power output (as measured by EMG) was reduced at the end of each 10 minute stimulation session even with only 8 stimuli per train (Figure 9).

Figure 10 summarizes the fatigue found in control, short-term (less than 2 weeks), and long-term (2-4 weeks) treated animals following electrical stimulation. The 8 pulse stimuli session resulted in about 10% reduction of power output in both control and treated animals. However, during the longer, 16 and 32 stimuli trains, the decrement in fatigue index was smaller in pyridostigmine treated rats. Specifically, the fatigue index

was, respectively, about 10% and 20% less in short- and long-term treated groups than in controls.

DISCUSSION

In this study we found a great variance at all times in serum AChE. This large interindividual variation was maintained following treatment with pyridostigmine even though the mean was significantly depressed (Figure 2; Table 1). The functional significance of interindividual variation in AChE is unknown but has been well documented (41).

Pyridostigmine is a reversible AChE inhibitor (41). We found some indication of tolerance developed to the drug treatment: AChE inhibition generally decreased as length of treatment increased. Studies involving long term treatment with antiChEs (or other agents that prolong the lifetime of the ACh molecule) have found tolerance via decreased sensitivity and number of postsynaptic ACh receptor-ion channel complexes (21-23) and changed presynaptic conduction as well (42). It may also be worth noting that the acute inhibition of AChE activity by pentobarbital has been shown to potentiate protection afforded by atropine and pralidoxime 2-PAM) against parathion poisoningin rats (43).

Our measures of behavior, physiology and morphology were all characterized by great variance among animals. This may be related to the great variance in serum AChE that we measured. In addition, motor activity or inactivity has been found to change AChE levels in muscle (44-46). Thus, the forced high exertion running itself might change the effect of the drug dosing.

Neuromuscular junctions from characteristically slow (soleus) or mixed type (gastrocnemius) muscles stained lightly and heavily for AChE, respectively. These findings are consistent with the literature on muscle fiber type and histochemical differences (47, 48).

Muscles from treated rats required more time to stain than simultaneously stained central muscles, possibly indicating less availability of AChE in muscle not only in serum. This should be confirmed with direct measures of AChE in muscle.

AChE could have affected synapse modeling but there were too few samples (total N = 113) to conclusively show that pyridostigmine treatment resulted in overall loss of neuromuscular synapses (Table 6; $p > 0.05$).

Different muscle fiber types appear to be differentially susceptible to the chronic antagonism of the antiChE pyridostigmine. Our histochemical findings (Table 5) would support the greater susceptibility of the type II,A (49), white, fast-twitch (50) fiber class (heavily stained for acid pre-incubated alkaline ATPase) rather than the respective type I, C and B, red, slow-twitch, lightly stained muscle fibers (51). The magnitude of this effect is notable, even with the small number of animals studied. In both soleus, a slow muscle, and gastrocnemius, a mixed muscle, no type II fibers were found after pyridostigmine treatment. Transformation of fast-twitch into slow-twitch fibers was demonstrated using

ultrastructural characteristics in rabbits exposed to low-frequency electrical stimulation (48, 52).

Both chronic chemical stimulation of muscle by antiChE and chronic electrochemical stimulation by direct electrical stimulation result in extended presence in neuromuscular junction of ACh released from the presynaptic nerve. Many changes have been found in muscle beyond those of the synaptic cleft following exposure to antiChE agents (19, 21-23). These changes might be part of the process of muscle type transformation. A transformation in the opposite direction from slow to fast fiber was found in rat soleus muscle following enforced disuse (46). It is now generally accepted (53) that the state of muscle is dynamic and plastic, responsive to activity demands. The concept of a rigid, immutable fiber type is antiquated. Relevant to our study, the state of muscle has been changed directly by exercise (54, 55). Furthermore, use (44) and disuse (46) themselves can change AChE activity in the muscle itself. Thus, converging influences of chemical antagonism, motor activity, and other individual variations could contribute to the large variance that we found amongst our animals on both behavioral and morphological measures.

Loss of synaptic axon terminals is a feature of AChE inhibition (56, 57). Our restricted survey showed only indications of a trend towards synapse loss following early pyridostigmine treatment (Table 6). This may be relative to change in muscle mass. After 14 days of pyridostigmine treatment, soleus:body weight ratio was greater than in control, while after longer treatment, this ratio was less than in control (Table 7), perhaps indicating a general wasting that ultimately affected muscle preferentially.

The neuromuscular apparatus showed a wide response to antiChE treatment with pyridostigmine and to intensive exercise. This emphasizes that the neuromuscular junction is also dynamic, capable of adaptive reorganization (58, 59), a phenomenon known to occur during aging (60, 61), in paralysis (58), and with increased neuromuscular activity due to stimulation (62, 63).

Signs of damage in the neuromuscular junction including the presence of damaged mitochondria may be an indirect drug effect, and should be quantified (e.g., see 51, 64, 65). Other studies (66, 67) found that exhaustive running produced marked swelling and disruption in muscle mitochondria, supporting the present findings.

A number of studies have indicated that structural abnormalities in muscle and/or neuromuscular apparatus develop subsequent to exposure to antiChE agents (5, 7, 9, 11, 13-17). Muscle damage appears to require a threshold exposure in time and dose to the antiChEs (8) and damage can be prevented by prior denervation (9). Thus, the origin of the damage may be set back in a chain of events stemming from the extended presence of ACh released in the neuromuscular synapse, which results in excessive activation of proteases due to the influx of Ca^{2+} at the motor endplate

(12, 17, 68). In consonance with this, the degenerative changes found in muscle fibers of pyridostigmine treated rats but not of running rats were focused at subjunctional areas.

The extent of necrosis of muscle and nerve and its duration appear to be time and activity dependent (13, 14). The extent and reversibility of damage may be related to induction of metabolizing enzymes of the antiChE (43, 69). This supports our finding that high intensity running combined with pyridostigmine treatment appeared to result in less muscle damage than this running alone (Figures 19 and 20), and may be due to greater induction of regenerative factors (Fig 21; 70) by the combined drug-exercise paradigm, for which similar signs have been found in rat muscle following ChE inhibition (70).

It is important to note here again that chronic treatment with pyridostigmine failed to maintain a constant level of serum AChE inhibition. Longer treatment generally resulted in less inhibition. These factors may prove important in planning antiChE pre-treatment strategies which account for time-activity profiles in determining risk:exposure:-benefits for combat performance.

A high variance on all behavioral measures was noted in an individual rat's performance from session to session, perhaps related to AChE level at the particular time of testing - not yet examined, but not apparently related to day-night differences in activity, which was taken into account. Following AChE inhibition with pyridostigmine, behavioral variance increased (paradigm II), and the rat's performance became less consistent. This might be a reflection of changes in AChE on the neuromuscular level, or could be related to induced postsynaptic activation and remodelling of the motor endplate (cf. 53, 58, 71), for which latter we have presented some morphological data.

The running behavior examined here was intensive and exhaustive (Table 2). About 50% of rats in treated and control groups failed on at least one occasion to complete the required 1-hour run. Another less strenuous study (26) showed that antiChEs at doses like our own that did not produce overt symptoms of cholinergic crisis did impair motor behaviors requiring coordination (e.g. hurdle stepping).

While there was generally significant overlap of scores of treated and control rats, within the first 3 days of running there was some indication that treated rats performed better than controls (Figure 3), probably due to the increased titre of ACh in the NMJ, similar to improved performance of myasthenic gravis patients treated with Mestanon, on other pyridostigmine derivatives (72).

While pyridostigmine does not cross the blood-brain barrier to any significant degree, even slight passage and slight changes in ACh brain levels may affect learning and memory retention between running sessions (73, 74). That doesn't seem to be the case. The performance of rats treated

with pyridostigmine progressively improved during the weeks of running (Figures 4 and 6) and also tended to improve within the session after 2-3 weeks of training (Table 4, Days 21 and 28).

Thus, chronic pyridostigmine treatment was not simply associated with a decrement in performance. Actually, after rats had run 30 minutes within the running hour, there was an indication that pyridostigmine improved endurance ($p < 0.05$, 2-way ANOVA, Table 3). During this period, treated rats in both collapse and non-collapse categories were able to maintain running pace and thus received significantly fewer shocks than respective non-treated runners ($p < 0.01$, Table 3). This improvement was particularly clear among intermittently trained runners (Figure 7).

After rats had run for 2 weeks, they attained a plateau in performance. Adding pyridostigmine at this time generally led to a decrement in performance (Figure 8). When pyridostigmine treatment was stopped, running performance generally improved, and without lingering drug effect. In contrast, rats that had been trained gradually, continued to perform better than other groups even when pyridostigmine was introduced.

These differences in "crash-course" training and gradual training have certain similarities with protein-deprived and normal rats, respectively (75). In addition, intensive exercise of protein-deprived rats led to a more pronounced loss of type 2 than of type 1 muscle fibers (75), similar to the present histochemical findings (Table 5).

Electrophysiological tests did not demonstrate deterioration in neuromuscular function after pyridostigmine treatment. Under the intense, short, repetitive stimulation program used, pyridostigmine had no noticeable deleterious effect. Perhaps there are subpopulations of susceptible muscle fibers (76) sparing others.

The aim of this stimulation program was to test neuromuscular apparatus fatigue resistance at its uppermost demand state; under such conditions even a minor drug effect might be prominent enough to be recorded (77). This same assumption led Foldes et al. (78) to use a similar pattern of high-frequency stimulation (100 Hz). Bursting motorneuron firing at 90 Hz has been recorded in animals (79); in humans short ballistic movements can produce rates even higher than 140 Hz (for review see 39). Repetitive stimulation below fusion frequencies for both fast and slow isolated motor units will usually be fatigue resistant (76). EMG recordings did show some decrement, the amplitude at the end of the train was decreased relative to the beginning; suggesting that some neuromuscular junctions could not maintain synaptic transmission at such a rate; however, the decrement was less in pyridostigmine treated thán in control rats.

At 100 Hz or less, neuromuscular transmission failures have been seen especially in fast fibers when stimulation was continuous (76); thus brief duration tetani repeated over long periods might better test fast unit performance. Indeed this was shown in Kugelberg's work (Kugelberg, 1973)

where fast units become more stable under high frequency, short train stimulation.

Our results indicate that pyridostigmine may be able to partially overcome neuromuscular junction impairment by effectively raising the amount of ACh available. Pyridostigmine is used for treatment of post-synaptic neuromuscular blockade (mainly myasthenia gravis). It is possible that neuromuscular failure in the fast units may have been partially overcome by the drug treatment.

All of the results of this study indicate the need for carefully-planned paradigms with regard to conclusions about the effects of drug treating and trainaing. Furthermore, other significant factors, unrelated to direct or indirect cholinergic effects, should be considered. Specifically relating to tissue damage and mitochondrial biogenesis are major changes in free radical concentrations of muscle following exercise to exhaustion (80). With continued work (81), the functional as well as morphological deficits will surely be defined.

CONCLUSIONS

This study indicates that the effect of pyridostigmine on performance tests during daily 60 minutes of exhaustive running in rats (at doses of pyridostigmine that produce an average of 40% inhibition in whole blood AChE activity) depends on the state of pretraining of the rat. No major behavioral deficits occur in pyridostigmine-treated rats in comparison to running controls, when they are simultaneously trained and treated for up to 2-4 weeks (paradigm I). When the rats are pretrained to run and then treated, the variance of their running performance increases markedly; that is, they perform less consistently (paradigm II). When the drug is stopped but running continued, performance generally improves, with no lingering drug effect (paradigm II). There is even some indication, despite the large variance in performance and small number of animals in this study, that treatment during the first 3 days of training improves performance and endurance (the ability to continue running at the end of the 60 minute period vs. the beginning).

These behavioral findings are consistent with acute electro-physiological studies using high frequency (100 Hz) short trains (8, 16, and 32 stimuli). The treated animals show less fatigue (defined as a lesser decrement measured from the triceps surae muscles of the leg) in EMG (electromyographic response) at the end of 10 minutes of intermittent stimulation, in comparison to nontreated control rats.

As anticipated from the literature, fine-structural examination of the gastrocnemius muscle revealed focal damage at random neuromuscular junctions and in the surrounding muscle fiber. However, few muscle fibers were affected relative to the total number of fibers examined. Moreover, increasing the treatment length appeared to have an inverse relationship to damage; the same appeared true for treated running rats. Further, the

neuromuscular junction showed an ability to adapt to the experimental conditions. In a dynamic manner, in response to treatment and running, the number of axon terminals per motor endplate changed. The result must be quantified by means of the ratio of axon terminal to endplate area, or immunochemical or radiochemical measures of receptor densities, number of nerve endings per muscle fiber, or area of AChE stain in the motor endplate.

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APPENDIX 1

Figures

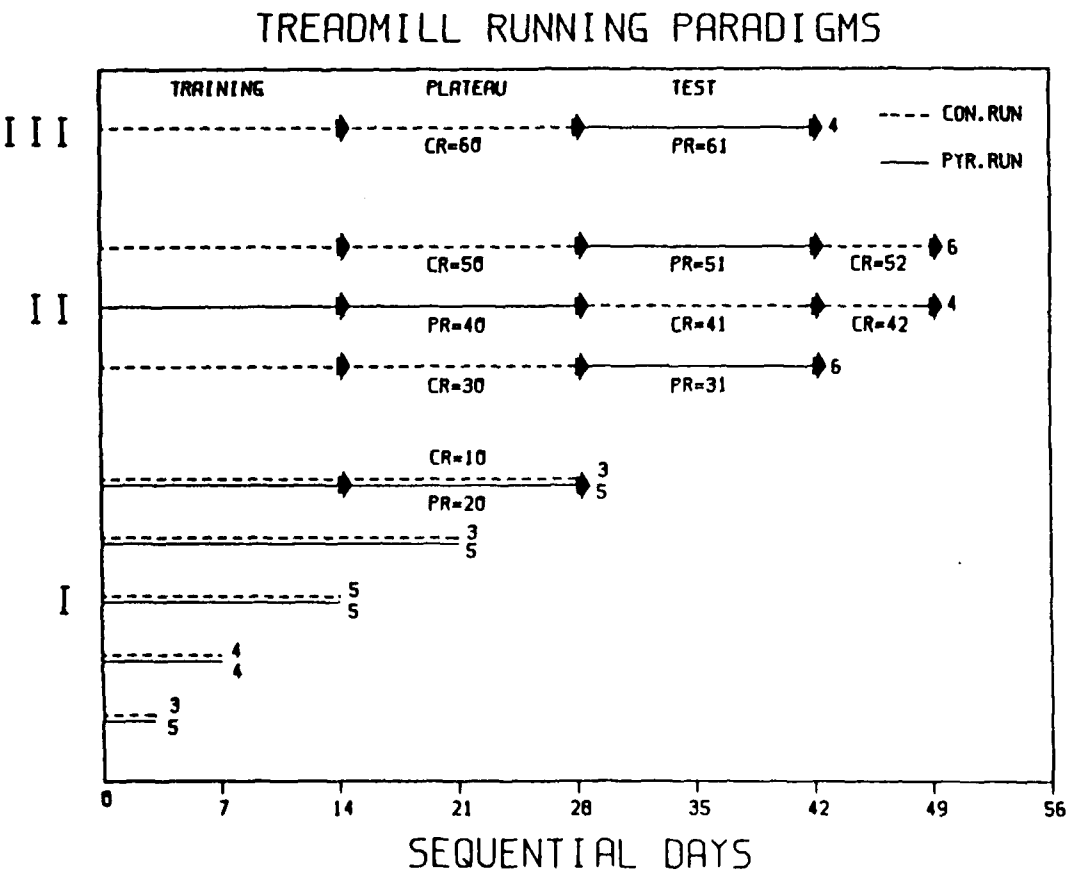

Figure 1

Treadmill running was divided into three major paradigms (I, II and III). All rats ran 1 hour per day with differences as noted. Paradigms I and II tested high speed running interrupted every 15 minutes for less than 1 minute (including groups 10, 20, 30 and 40). Treadmill speed averaged 28 m/min. Paradigm II tested groups run (50) throughout the hour without stopping but initial 15 minute treadmill speed averaged 20 m/min, then 28 m/min for 45 minutes. Paradigm II included groups pretained without drug (30, 50), then treated while still running (31; 51), as well as groups first treated and run (40, 51), then run without drug (groups 41; 52). Paradigm III tested gradual training over 14 days with running time and speed increasing up to 1 hour at 28 m/min (groups 60, 61). These rats also had a gradual warm-up over 15 minutes, then 28 m/min for 45 minutes without interruption (similar to 50, 52).

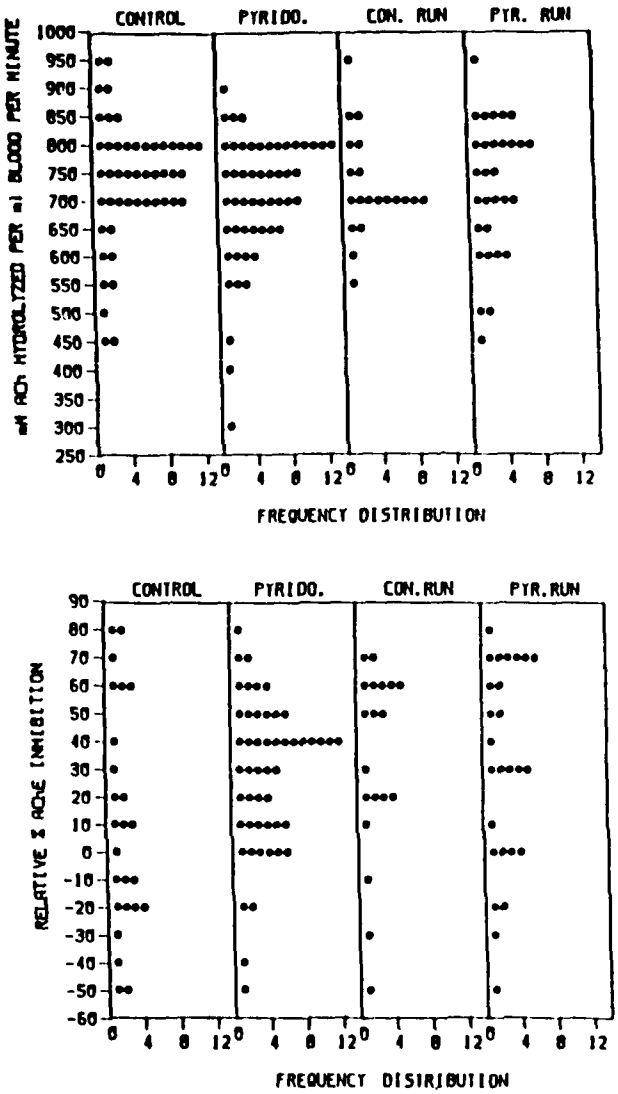

Figure 2

A. AChE activity measured in mmoles ACh hydrolyzed per ml whole blood per minute. Baseline response, prior to separation of rats into treatment groups. Overall: 750 ± 111 (N = 150).

B. Following pyridostigmine (Pyrido.) treatment and treatment with running (Pyr.Run), there was a significant inhibition in AChE activity ($p < 0.01$). Measurements were pooled for each rat relative to its own AChE activity prior to treatment. The control and untreated running groups (Con.Run) continued to show wide variance without significant inhibition.

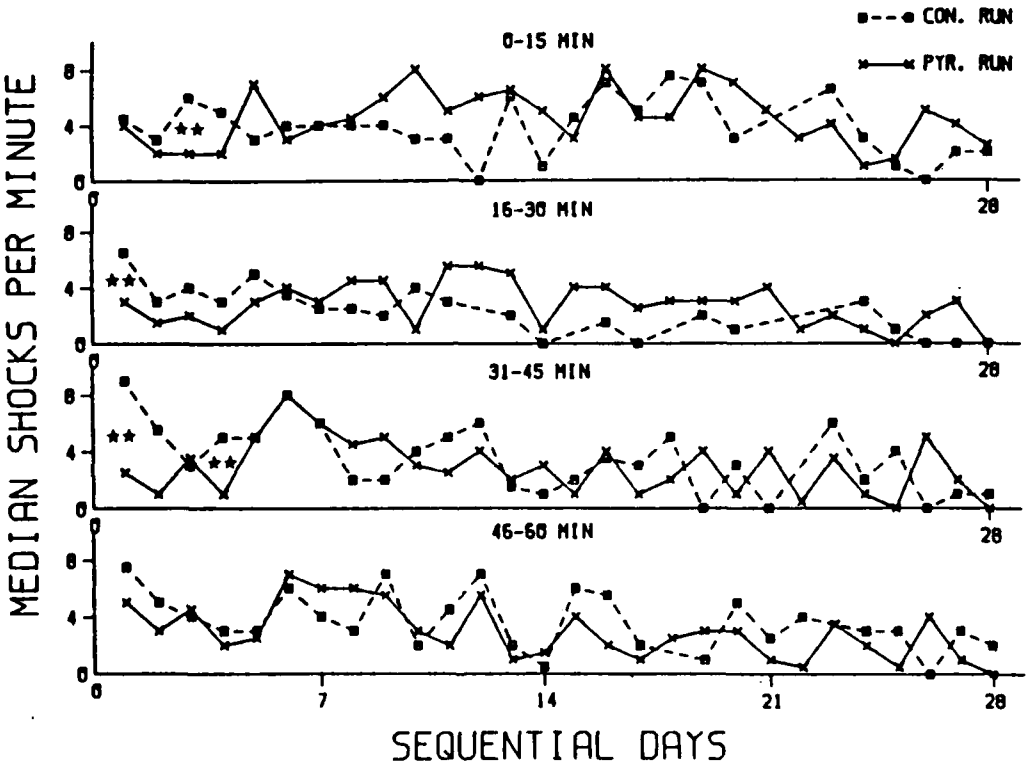

Figure 3

Running performance of pyridostigmine-treated and non-treated rats in paradigm I. To show differences within the hour of running, shocks per minute were meaned separately over 15 minute intervals.
* = $p < 0.05$; ** = $p < 0.01$; *** $p < 0.001$, for all figures and tables (Kruskall-Wallis ANOVA).

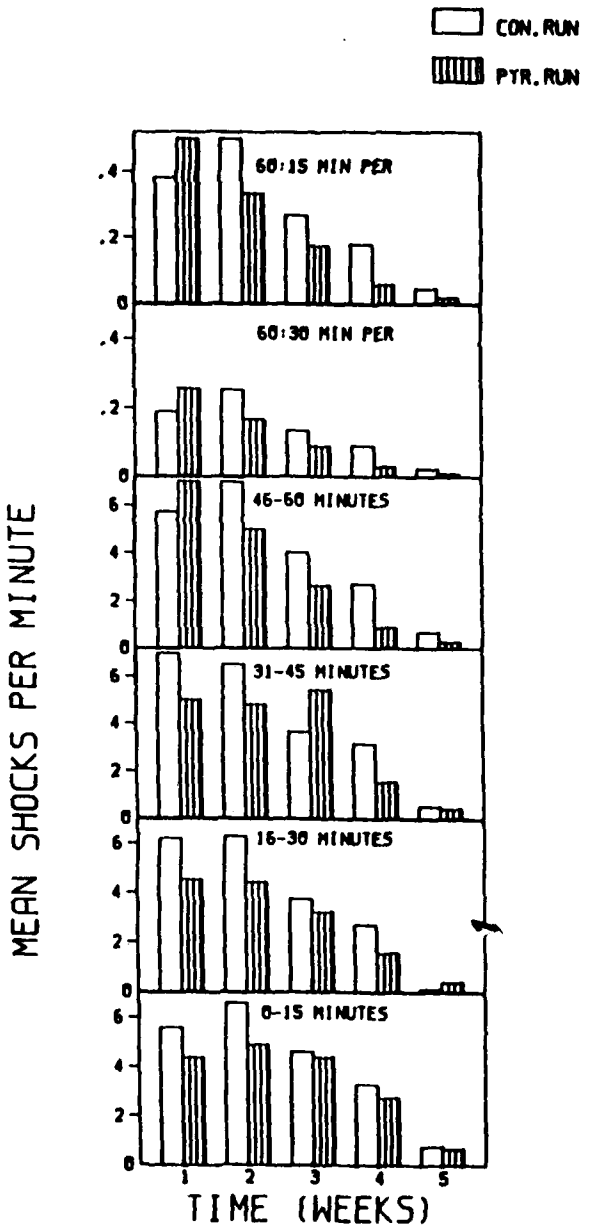

Figure 4

The mean shocks per minute obtained during running by treated vs. control rats, summed over weeks, indicate a consistent but insignificant ($p > 0.05$) decrease in shock index due to treatment. Both treated and control runners showed significant decreases in shocks per minute over weeks run ($p < 0.001$, MANOVA).

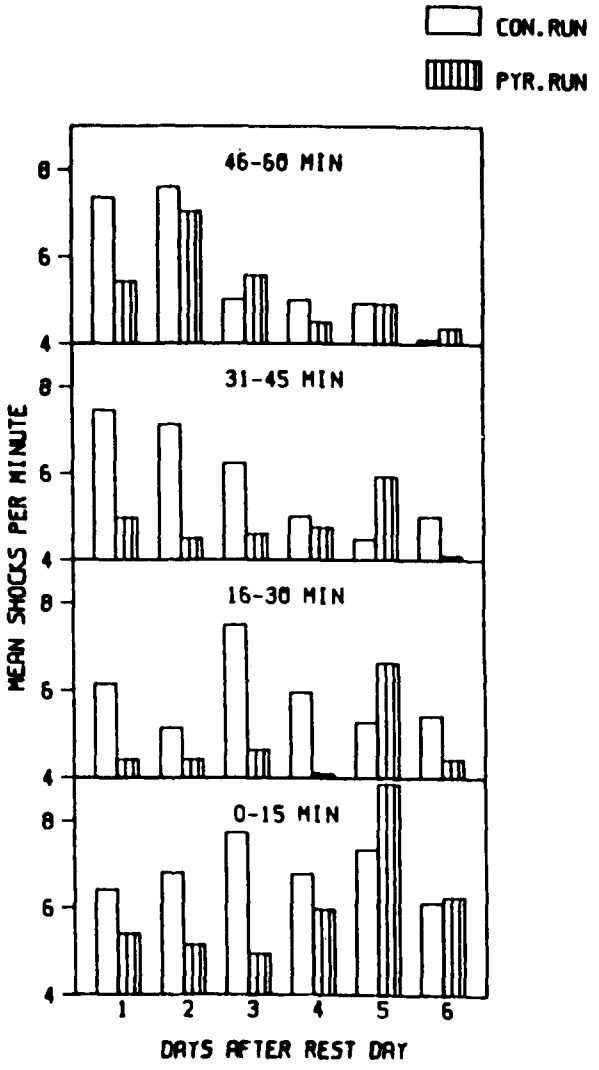

Figure 5

Rats rested from running 1 day in every 7. Analyzed by each day after the rest day for each 15 minute interval, the effect of treatment was clearly significant ($p < 0.001$, MANOVA), but that of rest day was only marginally significant ($p < 0.05$, MANOVA) when data were summed over all weeks of running.

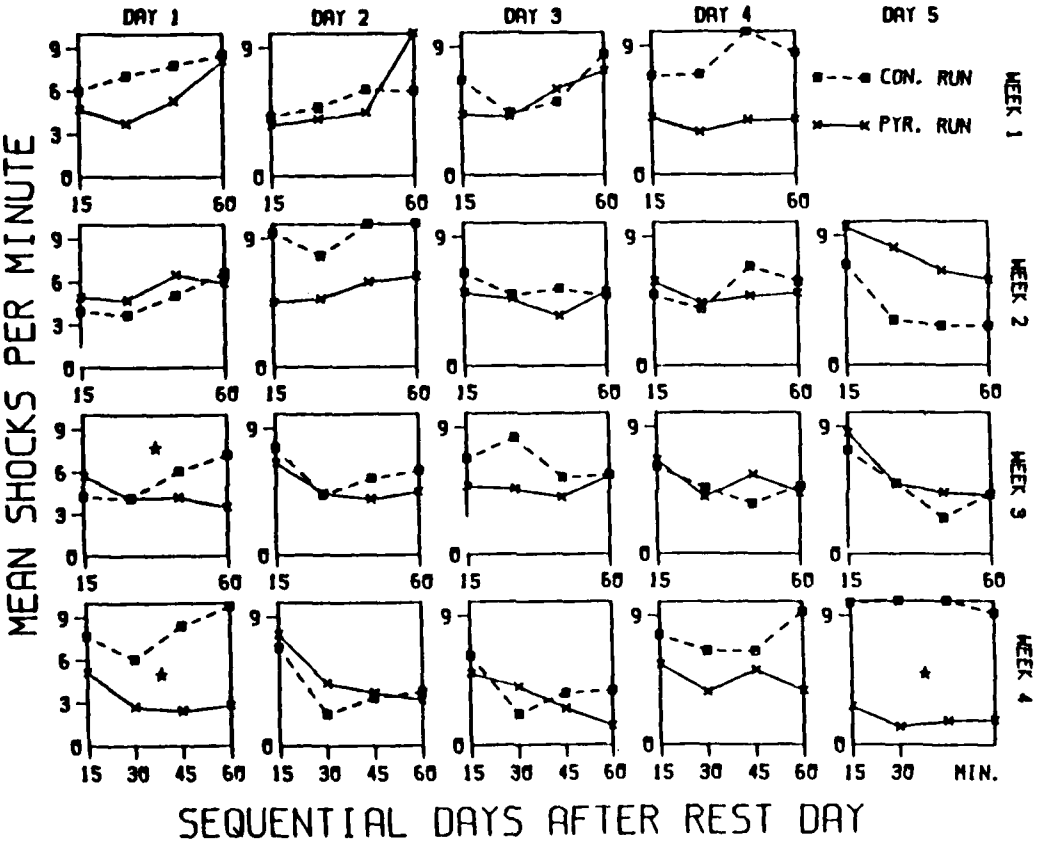

Figure 6

The mean shocks per minute obtained by control and treated running rats during each 15 minute interval on each day after rest for 4 weeks, averaged to show change in performance within the hour of running. Analyzed by median slope, the ANOVA showing only peripheral significance and notably after rats had been run for 4 weeks ($p < 0.05$).

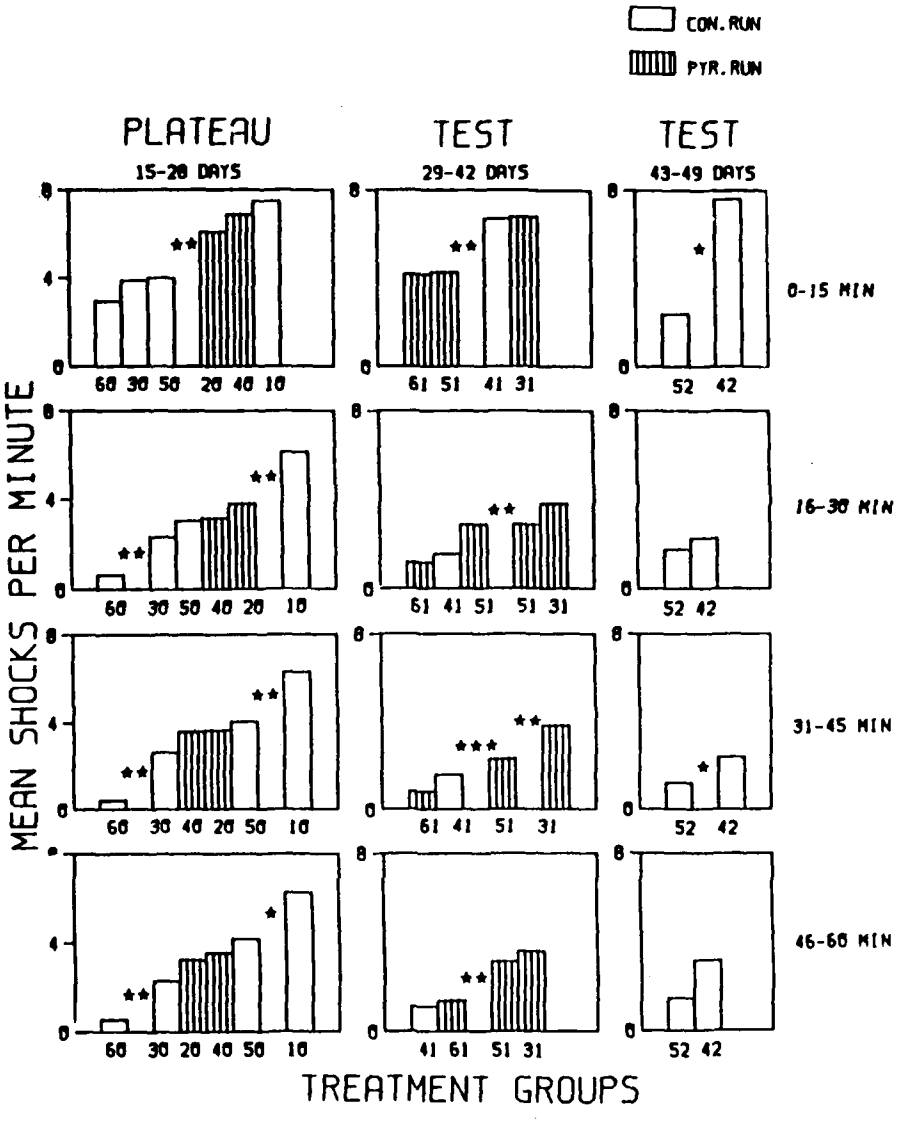

Figure 7

Running performance among all treatment groups[1] within plateau (15-28 days) and post-plateau periods.

1 Group designations (on abscissa) are as in Figure 1, paradigm of experiment. Groups with means that are significantly different from others are shown separated by asterisks, the number of which shows level of significance of difference. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$, also for Figure 8 (Kruskall-Wallis ANOVA, Student-Newman-Keuls range test).

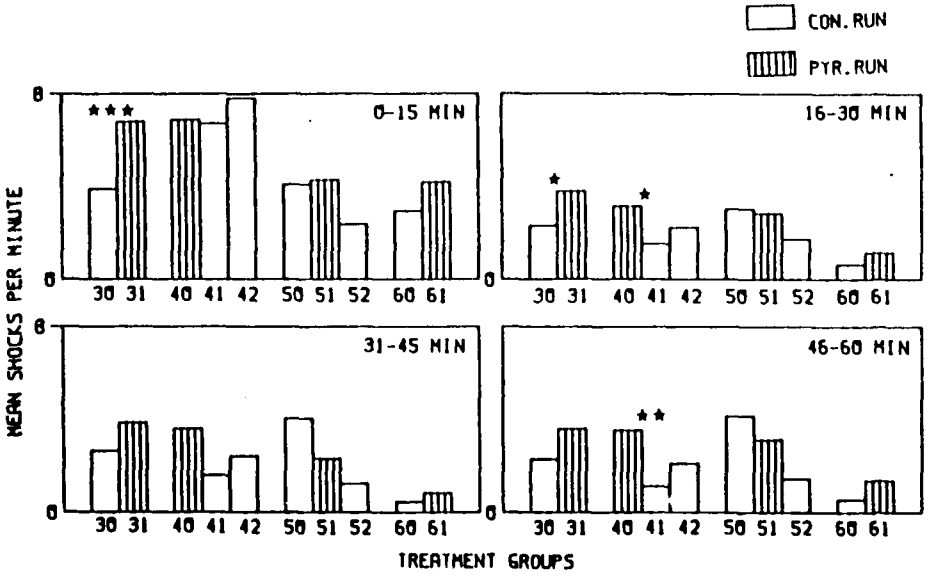

Figure 8

Comparison of running performance during and following the plateau period (days 15-28)[1] within each experimental group.

[1] Significant differences for paired comparisons are shown for periods 30-31; 40-42; 50-52; 60, 61, as in Figure 1, the Experimental Paradigm.

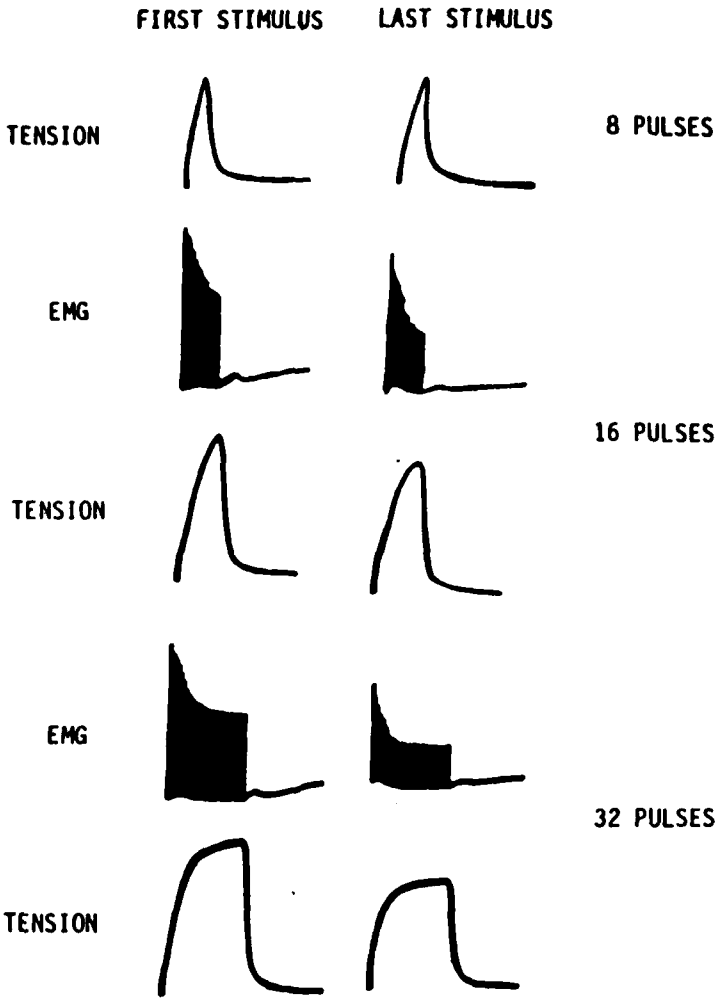

Figure 9

Muscle force, EMG and tension production, of triceps surae from anesthetized rats which had been treated with pyridostigmine or vehicle, was measured following high frequency (100 Hz), short burst (8, 16 or 32 stimuli), intermittent (one per 10 seconds) electrical stimulation of the sciatic nerve, in an in situ preparation.

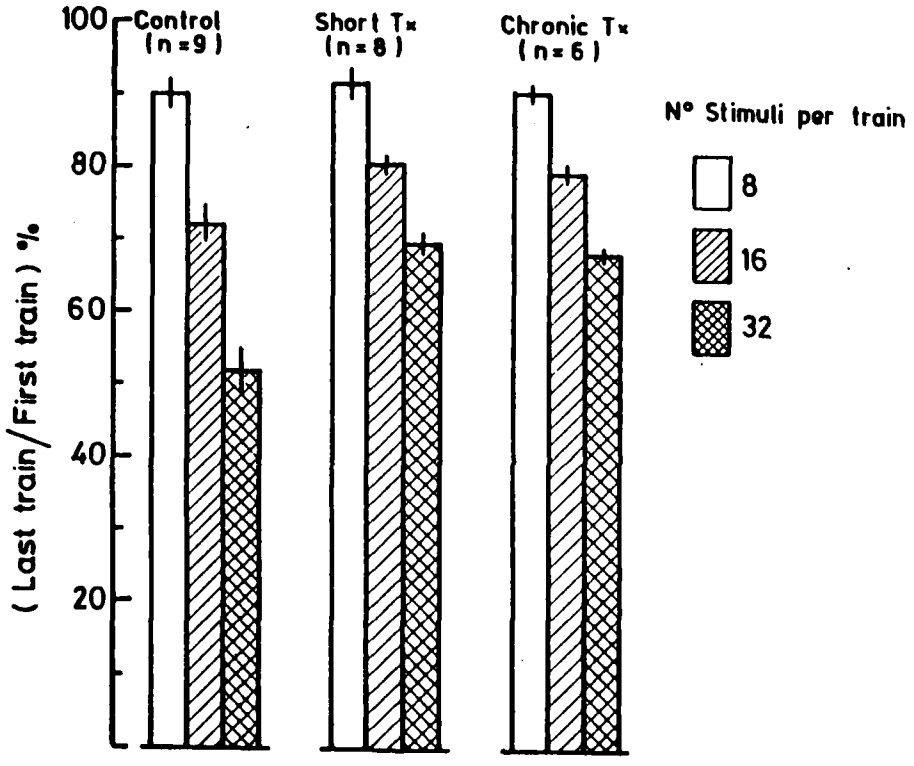

Figure 10

Relative decrement in EMG from the first to the last train of direct sciatic nerve intermittent tetanic stimulation in control, short-term (<2 weeks; Short Tx) and long-term (2-4 weeks; Chronic Tx) pyridostigmine-treated rats.

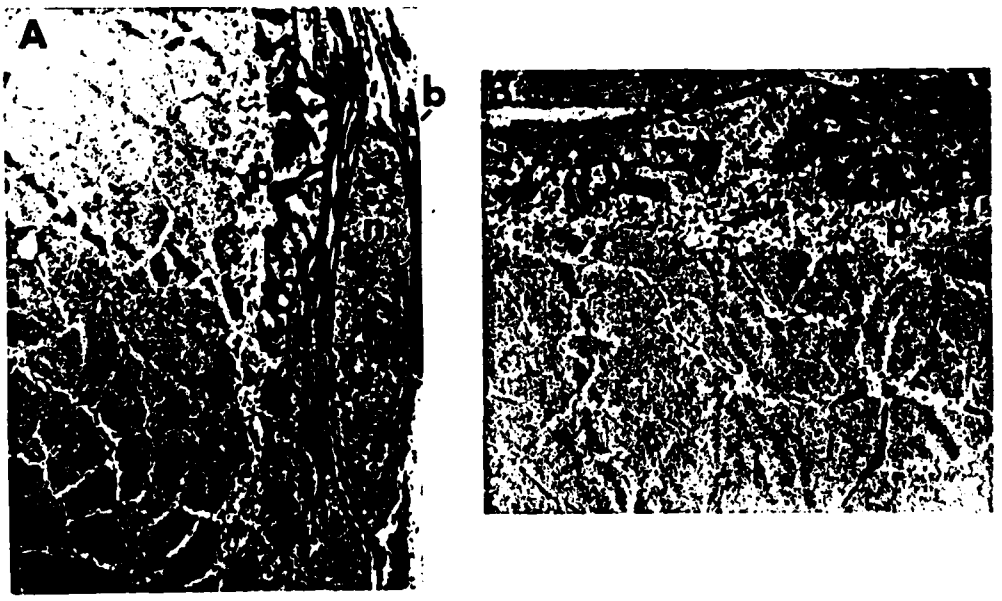

Figure 11

Electron micrograph, X-S, showing the postjunctional folds (p) and basal lamina (b) of a control gastrocnemius muscle filled with electron-dense AChE stain. Muscle nucleus (n) is seen below the basal laminal.

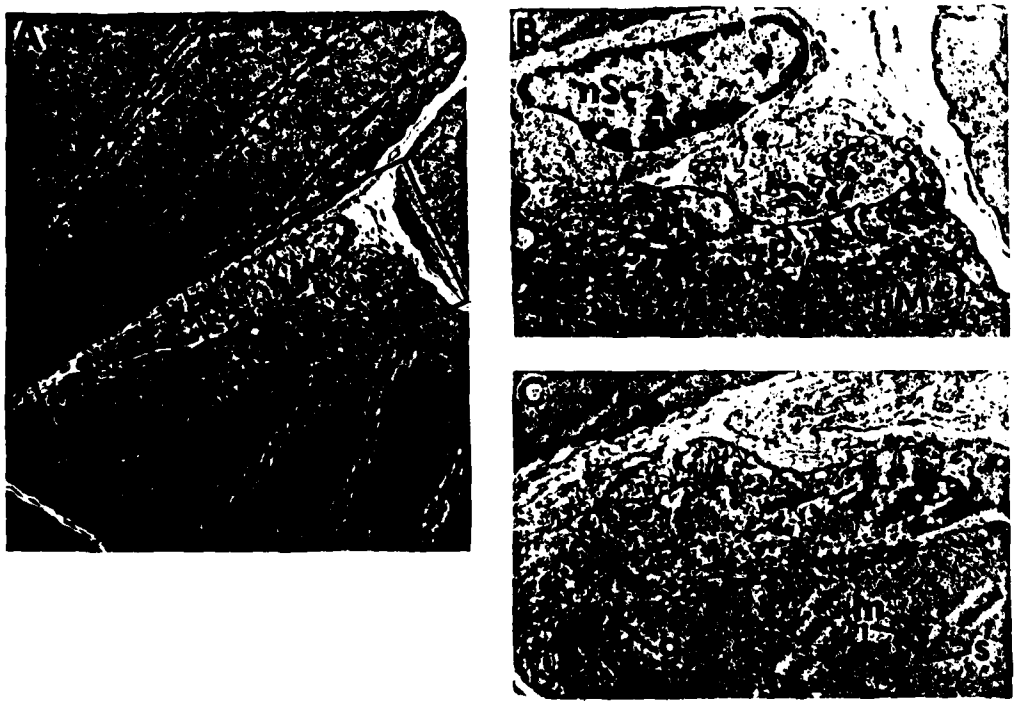

Figure 12

Electron micrograph, X-S, of a control gastrocnemius muscle. The muscle is indistinguished. A Schwann cell nucleus (nSc) is seen outside the basal lamina. The postjunctional folds (p) underlie axon terminals, below which is seen a nucleus of the muscle (nM).

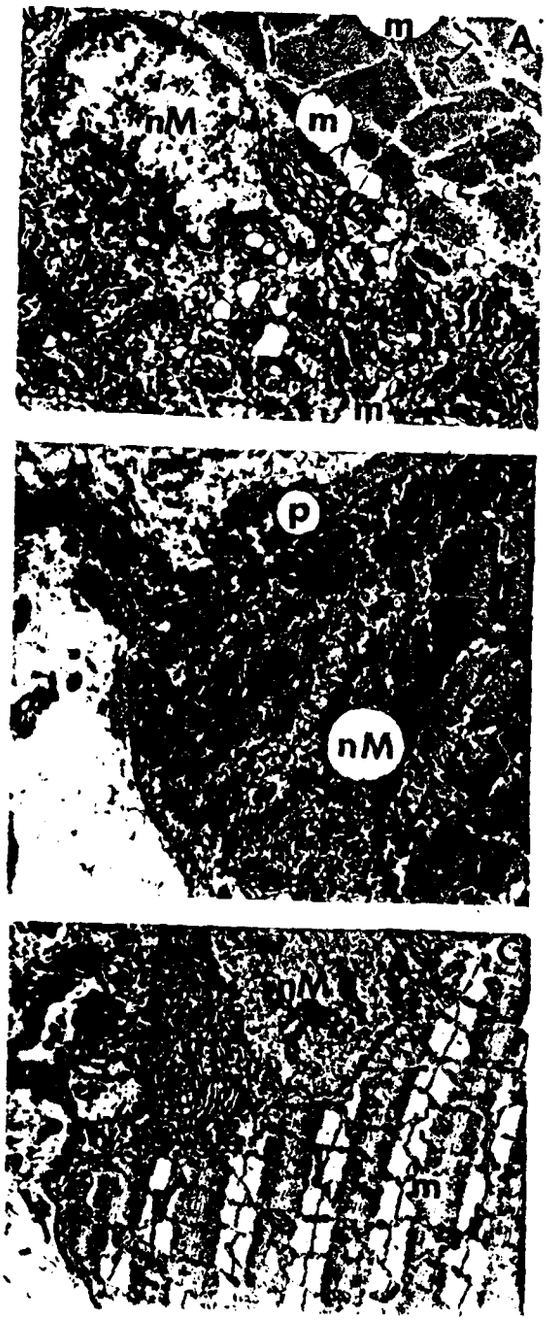

Figure 13

Electron micrographs, X-S, of gastrocnemius muscle of three 14-day pyridostigmine-treated rats. Note differences in extent of change within this treated group (A to C, most to least). In A, muscle mitrochondria (m) and sarcoplasmic reticulum (s) are distended and broken, but mitochondria (m) within the axon terminal (A) appear less affected. The subjunctional region is dense with vesicles and vacuoles. The axon terminal (A) in C is notably depleted of synaptic vesicles (v).

Figure 14

Electron micrographs, X-S, of gastrocnemius muscles of rats that were treated with pyridostigmine and also ran exhaustively for 1 hour per day for 14 days. Only one cystic body, or mitochondrial (m) remnant is seen in A-B; C-D is filled with them. Note variation in synaptic vesicle (v) density among the three axon terminals (A). An axon terminal is missing (small arrow), leaving an empty synaptic gutter in C.

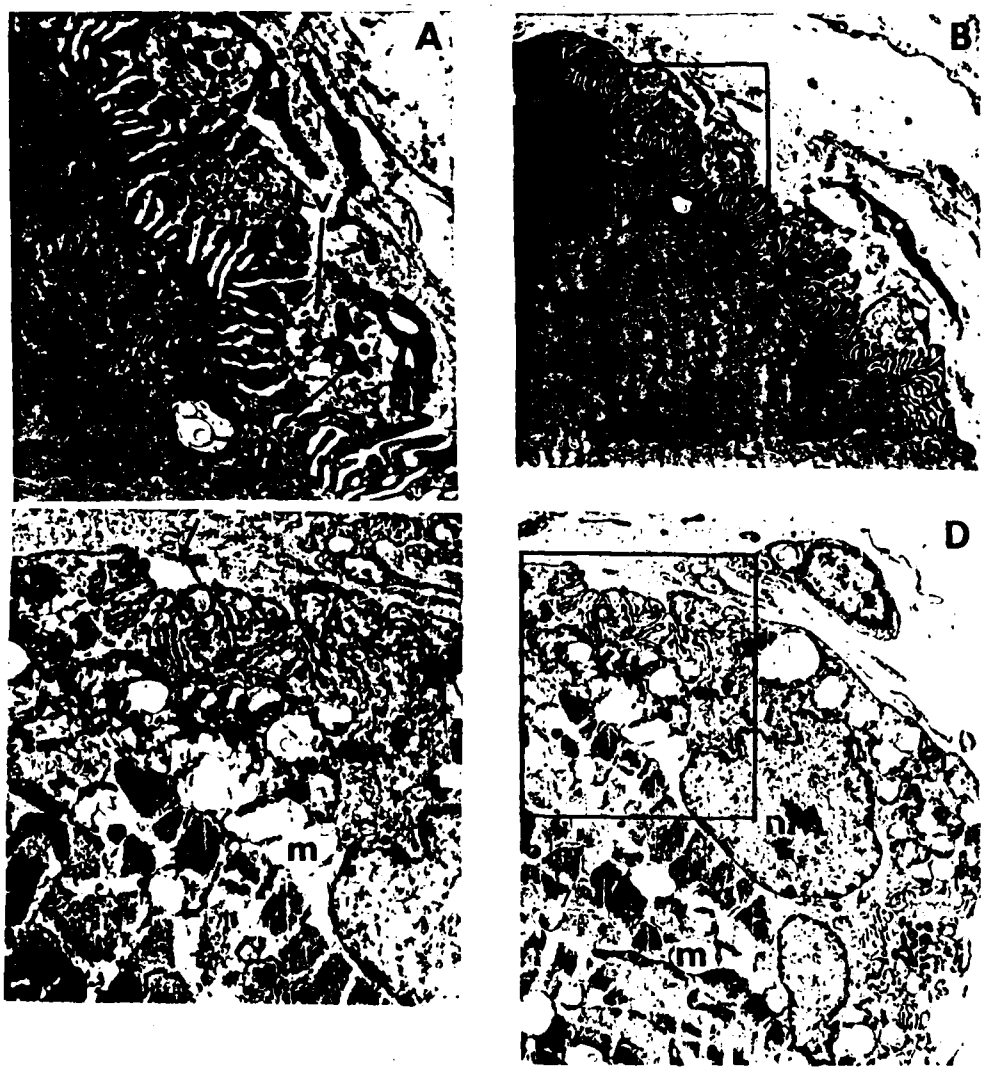

Figure 14

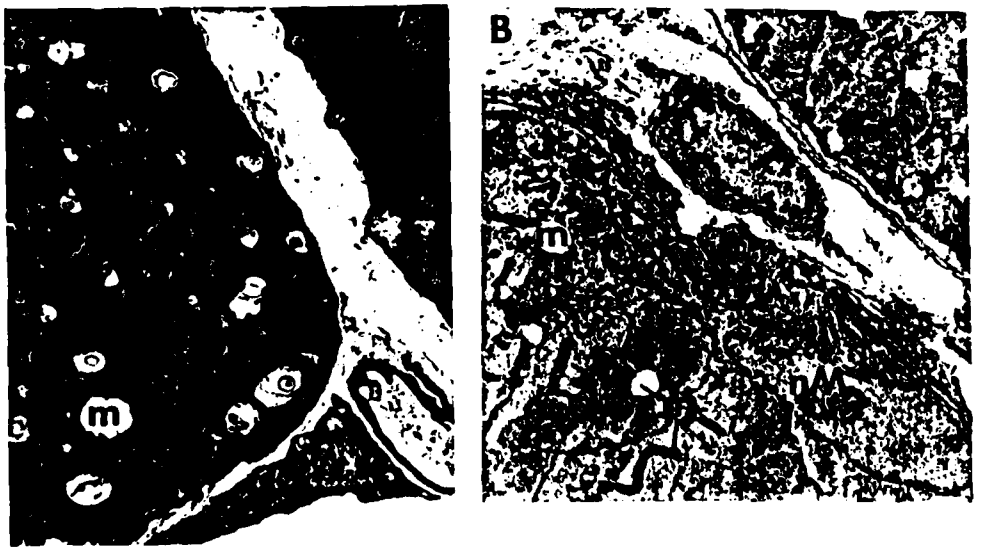

Figure 15

Electron micrograph, X-S, of 14-day daily runner. (Compare Figure 14.)
Note widespread or scattered mitochondrial (m) damage in muscle.

Figure 16

Electron micrographs, X-S, of gastrocnemius of 28-day pyridostigmine-treated rat. Note variation in synaptic morphology from nearly extruded in 16A to flat and broad in 16B (to "saddle bag"-shaped in Figure 18). Note dense-staining thickenings around tips (small arrows) of the postsynaptic folds (p) in 16B.

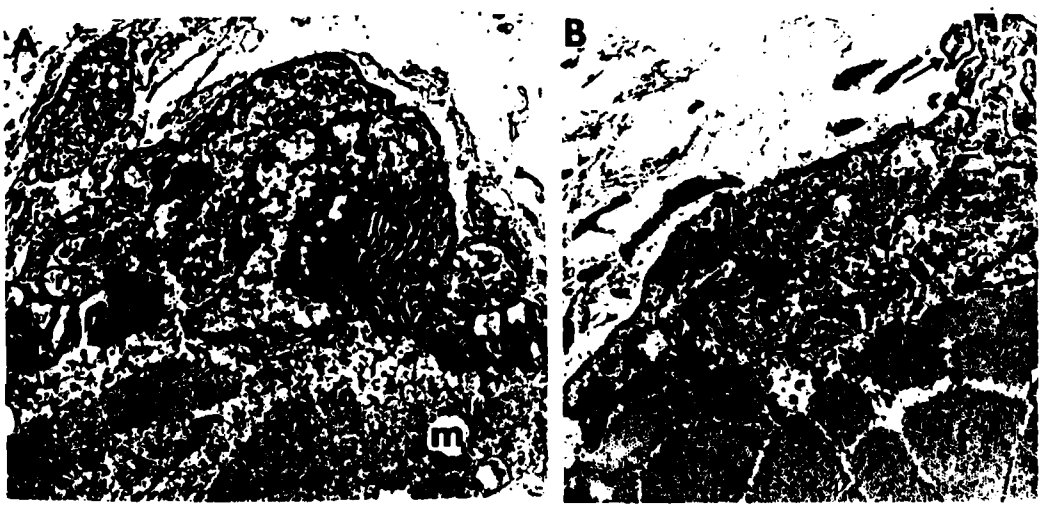

Figure 16

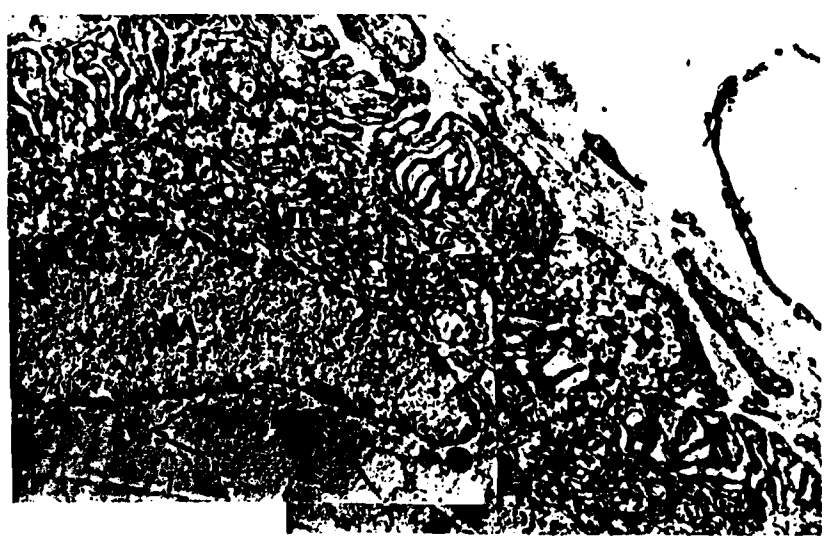

Figure 17

Electron micrograph, X-S, of gastrocnemius of a 28-day pyridostigmine-treated rat. Active zones can be distinguished within the axon terminal (stars) around the top of the postjunctional folds (p), while an adjacent nerve terminal appears retracted, and perhaps separated in part from the postjunctional folds by basal lamina.

Figure 18

Electron micrograph, X-S, of 28-day pyridostigmine rat gastrocnemius muscle. Synaptic vesicle clustering is not as distinct as in Figure 17, but there is some indication of synaptic vesicle fusion to the axon terminal membrane (small arrows). One of the axon terminals (A) appears retracted. The postjunctional folds are highly vesiculated with focal dense staining (large arrows). Rough endoplasmic reticulum (r) can be seen in subjunctional space, while muscle fibrils appear intact. Remnants of muscle mitochondria (m) are present.

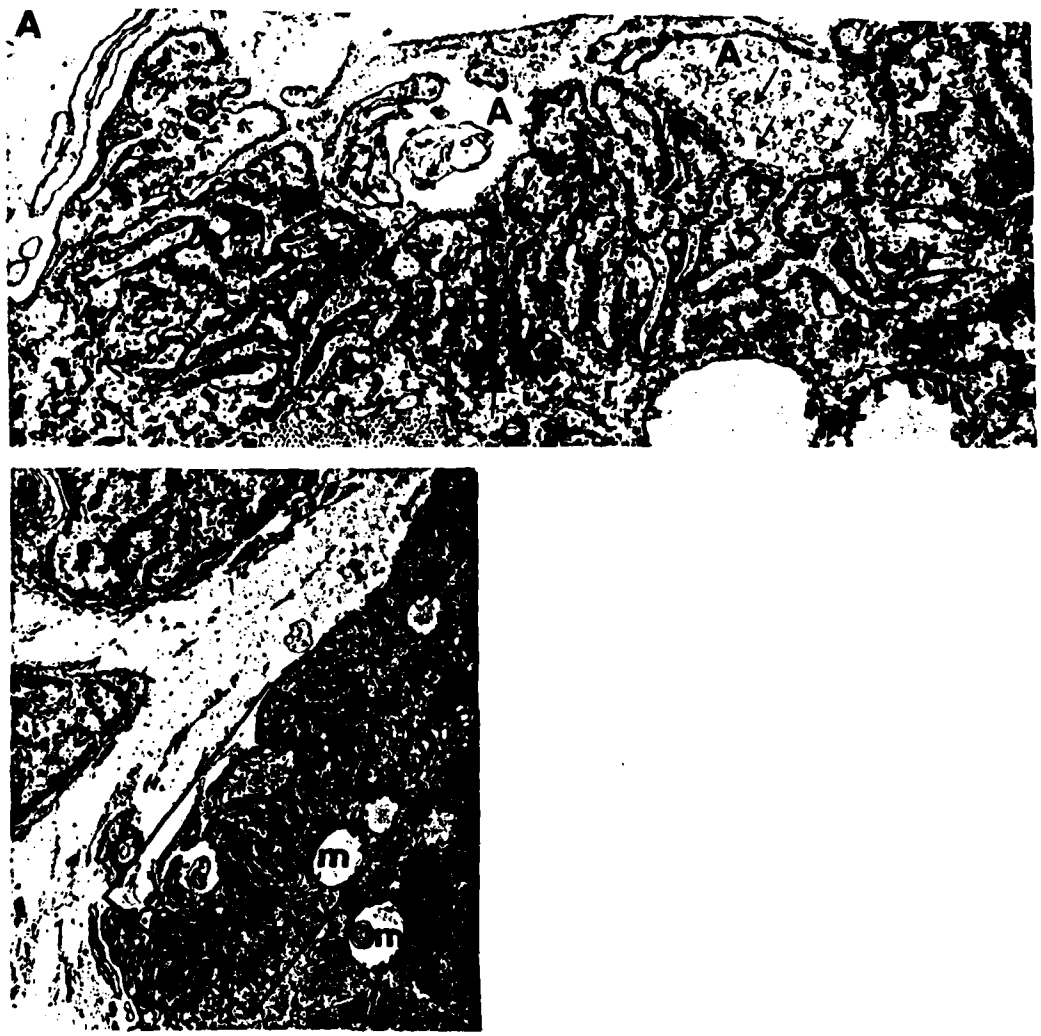

Figure 18

Figure 19

Electron micrograph, X-S, of gastrocnemius muscle of a 28-day pyridostigmine-treated running rat. Note the generally intact muscle. Dense staining vesicles and inclusions are scattered throughout the post-junctional folds (arrows). The sarcoplasmic reticulum (star) is distended in four places.

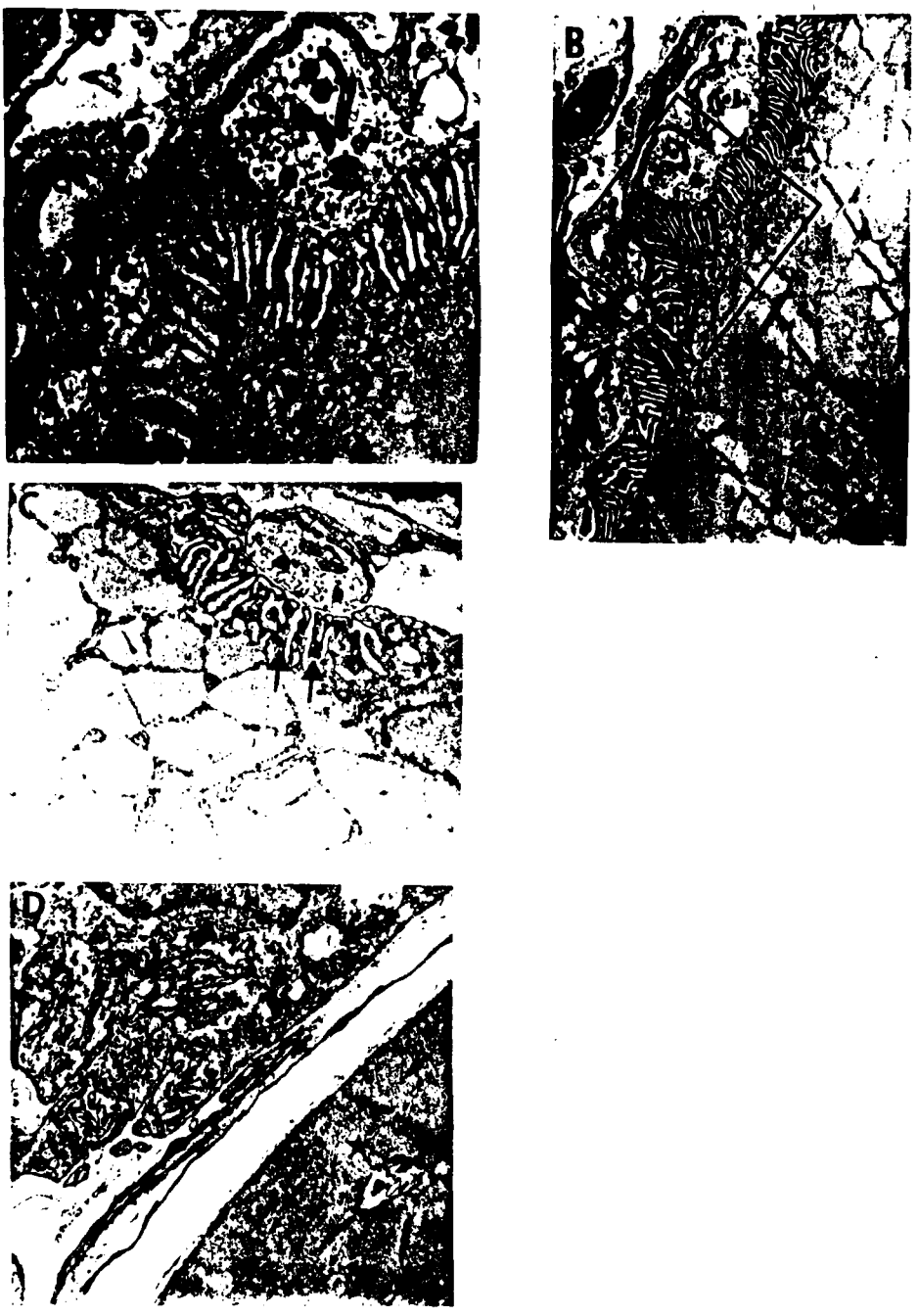

Figure 19

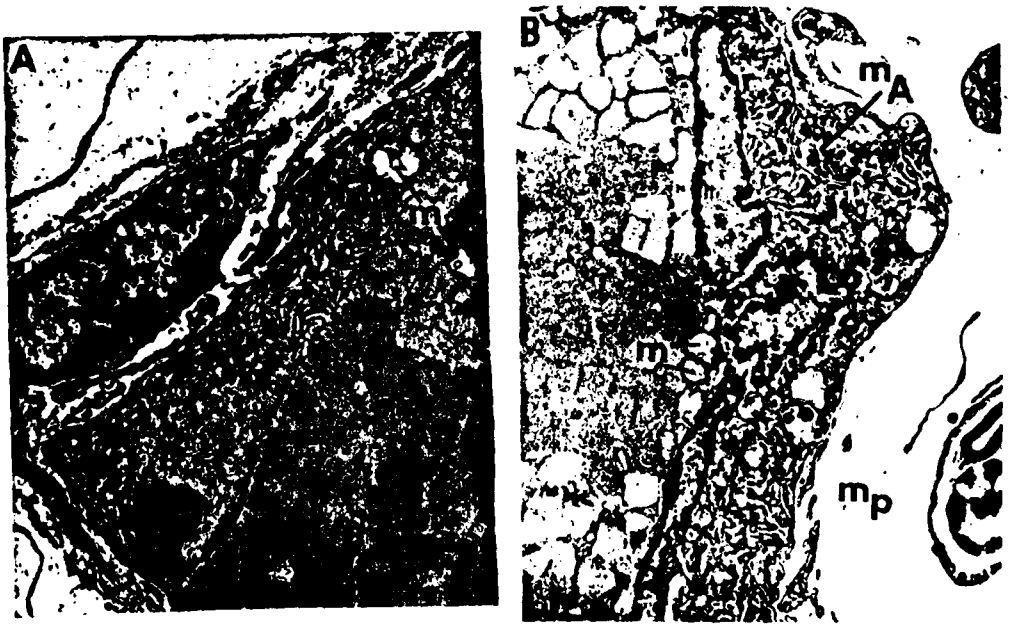

Figure 20

Electron micrograph, X-S, of gastrocnemius muscle of 28-day running, untreated rat. Abundant clustered, broken mitochondria (m) are present in and around the junctional space, but less so in the axon terminal (m_A). Damage varies markedly from specimen to specimen.

Figure 21

Electron micrograph. Non-junctional damage in gastrocnemius muscle of pyridostigmine-treated rats. Laminated inclusion bodies at the edge of the muscle fiber of (A) 28-day and (B) 14-day treated running rat. In (B) t-tubules (T) are seen, one filled with dense staining material. In (C), the membrane of a spindle fiber axon (A) is highly vesiculated and densely stained.

APPENDIX 2

Tables

TABLE 1

Acetylcholinesterase (AChE) Activity Before and After Pyridostigmine Treatment and Exhaustive Running

Group	Pre-Treatment		Post-Treatment		Relative average% inhibition
	AChE activity[1], mean ± s.d.	No. rats	AChE activity, mean ± s.d.	No. rats	
Control	767 ± 112 (766)[2]	49	602 ± 314 (650)	25	15
Treated	743 ± 116 (746)	56	489 ± 193 (457)	50	41**
Running (average 23 days)	766 ± 98 (743)	19	513 ± 230 (503)	19	23
Treated (average + running, 14 days)	748 ± 119 (756)	30	428 ± 214 (417)	26	47***

1 AChE activity was measured in mMole ACh hydrolyzed per ml of whole blood per minute.
ANOVA: ** = 0.01; *** = 0.001.

2 (Median value) of AChE activity.

TABLE 2

Summary of Measures of Collapse During Daily 60 Minute Running Sessions in Control and in Pyridostigmine-treated Rats

Measures of collapse in running rats	Untreated controls	Pyridostigmine-treated
Total number of rats that collapsed at least once	9	13
Percent collapsed rats of total number of rats run	50%	54%
Mean number of days each rat collapsed	2.7	2.3
Percent days of collapse of total days run	10%	4%
Median day of first collapse	5	3
Median day of collapse	10	8
Mean run time before collapse	33 minutes	42 minutes

TABLE 3

Cumulative Running Performance of Control and Pyridostigmine-treated Rats that Collapsed Versus Rats that Did Not Collapse Before Completing the 60 Minute Running Session

	Control		Treated	
	Number	Mean shocks ± s.d.	Number	Mean shocks ± s.d.
0-15 Minutes of Running Completed				
60 min run	138	6.1 ± 0.5	274	5.2 ± 0.3
<60 min run	30	10.6 ± 1.5	35	9.5 ± 0.9
		p<0.005[1]		p<0.001
16-30 Minutes of Running Completed				
60 min run	138	4.6 ± 0.4	274	4.0 ± 0.2
<60 min run	26	12.9 ± 1.9	34	9.4 ± 1.0
		p<0.001		p<0.001
31-45 Minutes of Running Completed				
60 min run	138	5.6 ± 0.5	274	4.5 ± 0.3
<60 min run	18	13.9 ± 2.3	21	8.3 ± 1.0
		p<0.01		p<0.001
46-60 Minutes of Running Completed				
60 min run	138	6.2 ± 0.5	273	4.9 ± 0.3
<60 min run	6	14.0 ± 6.2	11	22.6 ± 14.9
		n.s.[2]		n.s.

1 2-way ANOVA.

2 During the last 15 minutes of running (45-60), the comparison is not significant (n.s.) because too few run days are represented in the collapse category and the variance within the collapse category is great.

TABLE 4

Ranked Running Performance of Control and Pyridostigmine-treated Rats,

Across 15 Minute Periods Within the One Hour Running Session

	Number of rats	Minutes of running				Significance of difference in ranks
		0-15	16-30	31-45	46-60	
Day 1						
Treated	19	2.5	2.0	2.3	3.1	0.04
Control	10	2.4	2.1**[1]	2.6**	2.8	n.s.
Day 3						
Treated	19	2.1	1.9	2.8	3.2	0.02
Control	9	2.3**	2.0	2.9	2.8	n.s.
Day 7						
Treated	19	2.3	1.9	2.6	3.2	0.01
Control	8	2.0	2.5	2.9	2.6	n.s.
Day 14						
Treated	19	3.1	1.8	2.5	2.5	n.s.
Control	6	2.8	1.8	2.9	2.6	n.s.
Day 21						
Treated	7	3.9	2.1	2.6	1.4	0.04
Control	6	2.8	2.8**	2.9	2.6	n.s.
Day 28						
Treated	2	4.0	2.5	1.0	2.5	--
Control	3	3.0	1.7	2.3	3.0	--

[1] Significant differences in performance due to treatment are indicated by asterisks (**, $p < 0.01$; 1-way ANOVA).

TABLE 5

Relative Average Number of Muscle Fiber Types in Soleus and Gastrocnemius Muscles of Rats Treated with Pyridostigmine[a] or with Vehicle. Fiber Staining after Alkaline (pH 9.4) ATPase Preincubated at pH 4.3

Group	Dark (D)	% [b]	Intermediate (I)	%	Light (L)	%	N Fascicles	Ratio (%)[c] L:D
			SOLEUS					
Control	9.9± 5.4 (89)[d]	22	3.1±2.9 (28)	5	42.2±17 (380)	76	9	543± 33
	13.0±14.2 (91)	21	2.0±3.0 (14)	3	48.4±38.6 (229)	76	7	520± 28
Treated	10.2± 5.7 (82)	32	21.0±6.2 (168)	68	0 (0)	0	8	280±170
			GASTROCNEMIUS					
Control	19.9± 8.8 (179)	59	5.6±3.4 (50)	18	7.6± 3.8 (68)	24	9	37± 14
Treated	29.1±11.7 (262)	74	9.9±4.2 (89)	26	0 (0)	0	9	35 ± 7
	29.0± 3.7 (261)	76	9.0±2.4 (81)	24	0 (0)	0	9	31 ± 8

[a] Mice were treated with pyridostigmine for 28 days.

[b] % of total number fibers counted.

[c] Ratio is calculated as mean ± S.D. of light and dark fibers of all individual fascicles of each muscle.

[d] (N) = Number of individually counted muscle fibers in all fascicles combined.

TABLE 6

Mean Number of Axon Terminals per Synapse in Gastrocnemius Muscle of Rat after 0, 7, 14 and 28 Days of Running and Treatment with Pyridostigmine

Days of treatment	Group		
	Runner	Treated runner	Treated, nonrunner
7	2.42 ± 1.08[a] (12)[b]	-	1.33 ± 0.58 (3)
14	3.00 ± 1.44 (2)	2.56 ± 1.13 (10)	2.30 ± 0.95 (10)
28	2.00 ± 1.44 (2)	2.35 ± 1.27 (17)**	3.26 ± 0.77 (15)

Control[c]: 3.04 ± 1.13 (43)

[a] Mean ± s.d.

[b] Number of synapses counted.

[c] Nontreated, nonrunner.

** $p < 0.02$, difference from control.

TABLE 7

Mean (± s.d.) Ratio of Soleus Weight: Body Weight x 10000 in Rats After Chronic Treatment with Pyridostigmine and/or One Hour of Daily Exhaustive Running on a Treadmill

Treatment	14 days	21 days	28 days
Pyridostigmine	7.67 ± 0.13 (4)**	4.50 ± 0.14 (8)*	5.08 ± 0.53 (18)
Pyridostigmine + Running	6.49 ± 0.106 (8)	5.40 ± 0.49 (4)	6.54 ± 1.74 (6)
Running	6.11 ± 0.93 (6)	-	-
Non-treated + non-running	5.59 ± 1.64 (14)		
Probability levels of difference among groups on each day (1-way ANOVA)	0.01	0.06	0.02

* = 0.05; ** = 0.01; difference from control, Students T-test.

Rats were sacrificed at the end of the treatment period specified (N rats).

APPENDIX 3

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APPENDIX 4

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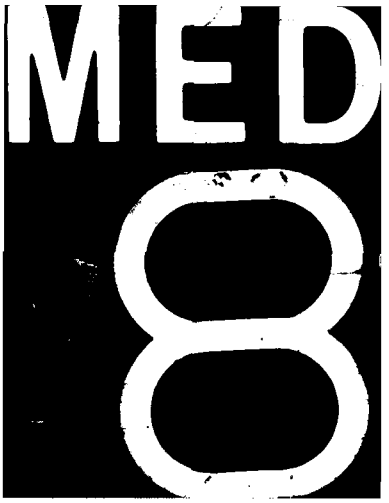
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